PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin E. Reubinoff, et al.	**Examiner:**	Deborah Crouch, PhD
Serial No.:	09/970,543	**Art Unit:**	1632
Filed:	4 October 2001	**Confirmation No:**	1839

For: EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION OF CLIVE NIELS SVENDSEN UNDER 37 C.F.R. §1.132

Sir:

I, Clive Niels Svendsen, hereby declare as follows:

1. I am currently the Professor of Anatomy and Neurology at the University of Wisconson-Madison, Madison.

2. I have worked in the field of human neural progenitor cells for over 10 years.

3. A true and correct copy of my curriculum vitae is attached hereto as Exhibit A.

4. I have been asked to provide my comments on the differences between human embryonic stem (hES)cells, hES cell derived neural progenitor cells (NPC), fetal/adult neural stem cells and fetal/adult neural stem cell derived NPC.

5. In particular, I have been asked to consider neural stem cells of the embryonic forebrain and compare those with hES cells and the neural progenitors that would derive from those cells.

6. However, any suggestion that neural progenitor cells isolated from human fetal cortex tissue at between 8-20 weeks of gestation, and human embryonic stem cells are identical is fundamentally not correct.

7. As stated above, I have been working on human neural progenitor cells derived from human fetal cortex for over 10 years. These cells can be expanded from the primary fetal cortex in culture as neurospheres. At early passages they produce high numbers of large pyramidal neurons and at later passages they produce high numbers of small GABAergic interneurons. I believe these cells are progenitor cells with a limited life span and are regionally specified at the time of dissection from the developing cortex to make cortical neurons.

8. I have specifically shown this regional specification in a recent publication in Ostenfeld et al, (2002) *Regional specification of rodent and human neurospheres*: Dev. Brain Res 134, 43 - 55.

9. A true and correct copy of the publication Ostenfeld et al, (2002) *Regional specification of rodent and human neurospheres*: Dev. Brain Res 134, 43 – 55 is attached as Exhibit B.

10. I have also discussed the concept of the cells following normal cortical development during their expansion in a review on Down syndrome work in Bhattacharyya and Svendsen; (2003) Human Neural Stem Cells: a new tool for studying cortical development in Down's syndrome, Genes Brain Behav, 2: 179-86.

11. A true and correct copy of Bhattacharyya and Svendsen; (2003) Human Neural Stem Cells: a new tool for studying cortical development in Down's syndrome, Genes Brain Behav, 2: 179-86 is attached as Exhibit C.

12. There is a fundamental difference between human neural progenitors isolated from the fetal cortex ($hNPC^{ctx}$) and human neural progenitors isolated from human ES cells ($hNPC^{ES}$). There are no reports of generating large projection neurons associated with the midbrain or spinal cord (i.e. dopamine neurons, motor neurons, serotonin neurons) from $hNPC^{ctx}$. This is because the progenitor isolated from the cortex at that

stage of development has already been specified to produce cortical tissues. I, and others, have tried extensively to modify $hNPC^{ctx}$ to produce dopamine neurons and have had little success. Although it is possible to get some tyrosine hydroxylase (a marker of dopamine neurons) expression through manipulations of the media these neurons are immature, do not express all the markers required for a dopamine neuron and do not survive transplantation or have functional effects in animal models. This issue is discussed in detail in my previous reviews on this topic in Svendsen et al (1999) *Human Neural Stem Cells: Isolation, Expansion and Transplation,* Brain Pathology, 9: 499-513 and Ostenfeld and Svendsen (2003) Recent advances in stem cell neurobiology, Adv Tech Stand Neurosurg 28, 3 - 89.

13. A true and correct copy of Svendsen et al (1999) *Human Neural Stem Cells: Isolation, Expansion and Transplation,* Brain Pathology, 9: 499-513 is attached as Exhibit D.

14. A true copy of the abstract of Ostenfeld and Svendsen (2003) *Recent advances in stem cell neurobiology,* Adv Tech Stand Neurosurg 28, 3 – 89 is attached as Exhibit E.

15. However, the situation is very different for $hNPC^{ES}$. Dopamine neurons with all of the correct physiological characteristics can be generated easily from $hNPC^{ES}$ as demonstrated in a number of recent papers. Furthermore, motor neurons with all of the characteristics of human motor neurons in the spinal cord can be generated from $hNPC^{ES}$ as shown in Li et al, (2005) *Specification of motoneurons from embryonic stem cells* Nat Bio. Published online 30 January 2005

16. A true and correct copy of Li et al, (2005) *Specification of motoneurons from embryonic stem cells* Nat Bio. Published online 30 January 2005 is attached as Exhibit F.

17. The reason for this is that hNPC derived from hES cells are fundamentally different to those derived from fetal cortex. They are at a very primitive stage of neural tube development which allows enormous plasticity with regard to the types of neuron they can produce as evidenced by these papers. Further studies using different protocols for differentiation will do doubt show a larger range of developmental possibilities from hES cell derived hNPC. Together with the artificial nature of hES cells as a starting source vs fetal cortex, the two hNPC types are in no way comparable and are distinctly different cells .

18. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Dated: 04/13/05

LIST OF EXHIBITS

EXHIBIT	ARTICLE
A	Curriculum Vitae
B	Ostenfeld et al, (2002) *Regional specification of rodent and human neurospheres*: Dev. Brain Res 134, 43 – 55
C	Bhattacharyya and Svendsen; (2003) Human Neural Stem Cells: a new tool for studying cortical development in Down's syndrome, Genes Brain Behav, 2: 179-86
D	Svendsen et al (1999) *Human Neural Stem Cells: Isolation, Expansion and Transplantation*, Brain Pathology, 9: 499-513
E	Ostenfeld and Svendsen (2003) *Recent advances in stem cell neurobiology*, Adv Tech Stand Neurosurg 28, 3 - 89
F	Li et al, (2005) *Specification of motoneurons from embryonic stem cells* Nat Bio. Published online 30 January 2005

EXHIBIT A

CURRICULUM VITAE

CLIVE NIELS SVENDSEN

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Personal

Born May 3, 1961, West Chinnock, UK

Education

1989-1991	Jesus College, University of Cambridge, Cambridge, UK, PhD, Dissertation: **"Regulation of cell death in basal forebrain cholinergic neurons".** *Supervisor: M.V. Sofroniew*
1980-1983	King's College, University of London, London, UK, B.Sc. in Zoology
1972-1979	King's Grammar School, Ottery St., Mary, Devon, UK

Professional Experience

2001-present	**Professor of Anatomy and Neurology,** University of Wisconsin-Madison, Madison
1997-2001	**Wellcome Career Development Research Fellow, Principle Investigator and Director of Training.** MRC Centre for Brain Repair, University of Cambridge
1995-1997	**Wellcome Career Development Research Fellow, Principle Investigator.** MRC Centre for Brain Repair, University of Cambridge Forvie Site, Robinson Way, Cambridge, CB2 2PY
1992-1995	**Postdoctoral Research Fellow.** Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB. *Dr. S.B. Dunnett's Laboratory.*
1991-1992	**European Manager,** ESA Analytical Ltd, 7 Cromwell Mews, St. Ives, Huntingdon, Cambs PE17 4HJ.
1988-1989	**Project Manager/Applications Specialist:** ESA Inc., 45 Wiggins Road, Bedford, MA, U.S.A. and Niko Bioscience, Nihonseimi Trade Centre Building 7-25-5. Nishigotanda, Shinagawa-ku, Tokyo 141, Japan.
1984-1988	**Neurochemist:** Brain Tissue Resource Centre Laboratory, McLean Hospital/Harvard Medical School, 115 Mill Street, Belmont, MA 02178, U.S.A.
1983-1984	**Research Assistant:** Brain Tissue Resource Centre, McLean Hospital/Harvard Medical School, 115 Mill Street, Belmont, MA 02178, U.S.A. *Supervisor - Professor E.D. Bird*
1979-1980	**Research Assistant**: Neurochemical Pharmacology Unit, Addenbrooke's Hospital, Cambridge, England. *Supervisor - Dr. M.N. Rossor*
1978	**Research Assistant**: Marine Biology Laboratory, Woods Hole, MA, U.S.A. *Supervisor - Professor J.Lash*

Honors and Awards

Wellcome Research Fellowship Award—1995
Included in the London Sunday Times: Top 100 People of the next 10 years—1998
Ernest Finch Memorial Lecture Award Recipient—2000
Featured in Nature Medicine Profile—2004

Grant/Program Reviewer

Wellcome Trust
Medical Research Council (UK)
Parkinson's Disease Society (UK)
Michael J. Fox Foundation
CNS Foundation
NIH study sections
Special emphasis panels
Invited to be member of NIH stem cell study section
Ad hoc phone reviews and study section attendance

Journal Reviewer

Nature
Science
Nature Neuroscience
Nature Biotechnology
Nature Medicine
J. Neuroscience
J. Neuroscience Research
J. Neurochem
PNAS
Experimental Neurology
Others

Editorial Boards

Experimental Neurology
Neurobiology of Disease

Scientific Advisory Boards

Michael J. Fox Foundation—New York, NY, USA
Children's Biological Solutions—Boston, MA, USA
Bright Foundation—Brookfield, WI, USA
BrainCells Inc—San Diego, CA, USA
Regenerative Medicine—Singapore

Meeting Organizer

September 2001, France; **"Neural Stem Cells: From Development to the Clinic"**. INSERM (Institut National de la Santé et de la Recherche Médicale)
October 9, 2003, UW-Madison, Madison, WI; **Translating Stem Cells and Growth Factors into the Clinic for PD and ALS,** *sponsored by the Department of Defense.* Acted as organizer to pull together some of the top researchers and government agency officials in the field to present in the areas of: basic stem cell and growth factor science, practical and regulation issues with translation of cell and growth factor therapy to the clinic, and moving forward towards clinical trials with stem cells and growth factors. 12 presenters and 70 in attendance.
September 11-14, 2005, Banbury Center, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. **Stem Cells and Axonal Regenerations: Strategies for the Treatment of ALS.** Co-organizer with Stephen Stittmatter (Yale University School of Medicine) and sponsored by The ALS Association.

Public Awareness of Science

Many talks to lay public on Parkinson's Disease; Stem Cells; Gene Therapy; and the Future of Medicine.

Research Grant Support Applied For

Svendsen (PI) *(RO1 application submitted March 1, 2005)* **NIH**
a-Synuclein Over-expression in Human Neural Stem Cells: with Bernard Schneider

Svendsen (PI) *(submitted March 24, 2005)* **NIH**
NIH Center for Excellence in Stem Cell Translational Research

Kiessling (PI), Svendsen (I) *(Pending)* (**NIH** U54 MH074418-01)
Molecular Libraries Screening Center for Human Biology

Current Research Grant Support

Svendsen (PI) *newly funded support* **NIH.**--$1,588,236.00
Stem Cell Research Training Program

Svendsen (PI) *(4/1/03-4/1/07)* **Department of Defense**--$1,694,183.00
Regulated GDNF Delivery in Vivo Using Neural Stem Cells

R01 NS045143 Dempsey (PI) Svendsen (Co-PI) *(1/15/03-12/31/07)* **NIH**--$649,002.00
Neurogenesis after stroke: effect of growth factors

Gamm (PI), Svendsen (Mentor) *(02/01/04 to 01/31/09)* **NIH**-- $332,101.00
Culture And Transplantation Of Human Retinal Stem Cells

Svendsen (PI), Mitchell (Co-PI) (1/31/05 to 1/30/06) **The ALS Association**--$240,000.00
Combined Delivery of Growth Factors and Astrocytes as a Potential ALS Treatment

Svendsen (PI) *(3/01/02-7/05)* **Michael J. Fox Foundation for Parkinson's Research**-- $396,478.00
Banking of Dopamine Neuronal Production from Human Neural Stem Cells

Svendsen (PI) *(1/01/05-1/06)* **Michael J. Fox Foundation for Parkinson's Research**
Comparison of Amgen human recombinant GDNF with human glycosylated neural stem cell derived GDNF

Zhang (PI) Svendsen (Co-PI)*(1/01/05-1/01/07)* **Michael J. Fox Foundation for Parkinson's Research**
Combined stem cell transplant and growth factor therapy for Parkinson's disease

Svendsen (PI) *(1/04/04-31/03/06)* **Kinetics Foundation**--$250,000.00
Generation of monkey ES cells and human neural stem cells for use in primate models of PD.

Svendsen (PI) *(01/01/05 to 12/31/05)* **American Heart Association** -- $26,000.00
Using Genetically Modified Neural Stem Cells to Repair the Damaged Rat Brain of Stroke

Svendsen (PI) *(7/20/04 to indefinite)* **Northwestern Mutual Foundation**--$50,000.00
GDNF production from human neural stem cells for Parkinson's Disease.

Svendsen (PI) *(10/30/03 to indefinite)* **Sigma-Aldrich**-- $72,750.00
Development of a culture medium for the expansion of human fetal neural stem cells in neurosphere.

Previous Research Grants (since 2000)

Svendsen (PI) and Zhang (CoPI) *(1/1/02 – 1/1/05)* **The ALS Association**- $426,120.00
Stem Cell and Therapy for ALS

Svendsen(PI) *(4/1/03-12/31/04)* **CNS Foundation**--$65,000.00
Generation of neural stem cells for transplantation

Svendsen (PI) *(6/1/02-5/31/03)* **FRAXA Research Foundation**--$50,000.00
Generating Human Neurons Carrying the Fragile X Mutation

S. Chadran and C.N. Svendsen (Sponsor) *(4/99-4/03)* **MRC Clinician Scientist Fellowship**
An In Vitro And In Vivo Study Of The Oligodendrocyte Lineage And Myelinating Potential Of Human Stem Cell Derived Oligodendrocytes

Svendsen (PI) and Caldwell *(9/99-8/02)* **Parkinson's Disease Society Medical Research Grant**
Human Neural Stem Cells As A Source Of Tissue For Cell Therapy In Parkinson's Disease

Svendsen (PI) *(4/1/95-9/30/00)* **Wellcome Trust Research Career Development Award**
The long term objective of this project was to develop novel methods of growing and transplanting human neural stem cells for cell replacement therapy programs. In addition, the molecular mechanisms underlying neural cell proliferation and differentiation were explored. The grants below supplement this core award to Svendsen.

Research Training

Past post-doctoral fellow and graduate students:
Borris Haupt
Rebekah Jakel
Soshana Behrstock
Anita Bhattacharyya
Masatoshi Suzuki
David Gamm (mentor for NIH grant)
M. Caldwell
J. Labelle
Telma Schwindt

Present post-doctoral fellow and graduate students:
Hung-Jung Kim
Aaron Nelson
Sandra Klein
Bernard Schneider
David Gamm
Allison Ebert
Shengli Xu

PhD dissertation committees
Aimee Ardoldussen
Daniel O'Bryan
Jim Windelborn
Matthew Pankratz
Sandra Klein

PhD Examinations
Served as external or internal examiner for 9 PhD dissertations

Teaching

2000-present	Taught course at UW-Madison including Biocore 2003 and many individual lectures for programs. Currently have 3 graduate students from the Neuroscience Training Program working in the lab. Director of training for NIH
1995-2000	stem cell training grant. External and internal examiner for PhD students. Supervisor for PhD and part II
1992-1994	students. Director of Training at the BRC for five years. Lecture series in the Medical Aspects of Neurobiology course to2nd year Cambridge medical students. Supervision of 2nd year medical students Part II
1990-1992	projects. (2 students per year). Supervision of 2nd year medical students at Emmanuelle College, Cambridge University for the neurobiology course.
1989-1992	Supervision of Part II neuroscience projects (2 students)
1984-1988	Supervision of 2nd year medical students in the Department of Anatomy, Cambridge University. Neuroanatomy practicals. Training and supervision of summer students from various colleges in Boston, MA. Taught basic neurochemistry, HPLC methodology and laboratory procedures.

Current Committees

Member of the Wisconsin Stem Cell Research Program executive committee
Member of the Society for Neuroscience
Member of the British Neuroscience Association
Group Member of NECTAR (European Neural Transplant Organization)

Patents

"Use of human neural stem cells secreting *GDNF* for treatment of Parkinson's and other neurodegenerative diseases"—patent applied for December 11, 2003

Bibliography

Book Chapters

1. Svendsen CN, Langlais PJ, Benes FM, Bird ED. (1986) Monoamine levels in the left and right amygdaloid complex of schizophrenic post-mortem brain tissue. In: *Biological Psychiatry* 1985, Shagass, C. *et al*, eds. Elsevier Science Publishing Co., Amsterdam. pp1115-1118
2. Sofroniew MV, Isacson O and Svendsen CN. (1990) Changes in basal forebrain cholinergic systems following excitotoxic cell death in the hippocampus and cerebral neocortex. *Behavioural Pharmacology Series*, Plenum Press.
3. Matson WR, Bouckoms A, Svendsen, CN, Beal MF and Bird ED. (1990) Generating and controlling multiperameter data bases for biochemical correlates of disorders. *Basic Clinical and Therapeutic Aspects of Alzheimer's and Parkinson's Disease*, Volume 1. Plenum Press, New York, 513-516.
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9. Chadran, S. and Svendsen, C.N. (1999) Neural Stem cells for Transplantation. In .Neural

Transplantation Methods. (Eds) Dunnett, S.B. Boulton, A..A. and Baker, G.B., pp41-54.

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11. Rosser, A.E., Ostenfeld, T. and Svendsen, C.N. (2000) Invited Commentary: Treatment of diseases of the CNS using encapsulated cells , by Hottinger, A.F. and Aebischer, P. In Advances and technical standards in neurosurgery, 26: 125-127
12. Behrstock, S. and Svendsen, C.N. (2003) Neural stem cells. Nature Encyclopedia of the Human Genome. 4: 298-302. Nature Publishing Group.

Reviews and Commentaries

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15. Dunnett S.B and Svendsen C.N. (1993) Huntington's disease: animal models and transplantation repair. *Current Opinions in Neurobiology,* **3**:790-796.
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38. Svendsen, C.N. and Langston, J. (2004) Stem cells for Parkinson's Disease and ALS: Replacement or protection? *Nature Medicine*, **10**; 224-225
39. Behrstock, S. and Svendsen, C.N. (2004) Combining growth factors, stem cells, and gene therapy for the aging brain. *Ann N Y Acad Sci,* **1019**:5-14

Primary Peer Reviewed Research Papers

38 Lash JW, Ovadia M, Parker CH, Svendsen CN. (1979) Tidal rhythm and tissue organisation in the neuronal gland of Ciona intestinalis: correlates with cellulose and fibronectin. *Biol. Bull.* **157**: 378.
39 Rossor MN, Svendsen CN, Hunt SP, Mountjoy CQ, Roth M, Iversen LL. (1982) The substantia innominata in Alzheimer's disease: an histochemical and biochemical study of cholinergic marker enzymes. *Neurosci. Lett.* **28**: 217-222.
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43 Rasool CG, Svendsen CN, Selkoe DJ. (1986) Neurofibrillary degeneration of cholinergic and non-cholinergic neurones of the basal forebrain in Alzheimer's disease. *Ann. Neurol.* **20**: 482-488.
44 Svendsen CN, Bird ED. (1986) HPLC with electrochemical detection to measure chlorpromazine, thioridazine and metabolites in human brain. *Psychopharmacology* **90**: 316-321.
45 Beal MF, Mazurek MF, Svendsen CN, Bird ED, Martin JB. (1986) Widespread reduced somatostatin-like immunoreactivity in the cerebral cortex in Alzheimer's disease. *Ann. Neurol.* **20**: 489-495.
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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin E. Reubinoff, et al.	**Examiner:**	Deborah Crouch, PhD
Serial No.:	09/970,543	**Art Unit:**	1632
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DECLARATION OF CLIVE NIELS SVENDSEN UNDER 37 C.F.R. §1.132

This is the exhibit marked as "Exhibit B" referred to in the Declaration of **CLIVE NIELS SVENDSEN**

By: _____ Dated: 04/13/05

EXHIBIT B

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Interactive report

Regional specification of rodent and human neurospheres

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Abstract

Neural precursor cells were isolated from various regions of the developing rat and human brain and grown in culture as aggregates termed neurospheres. We asked whether cells within human and rodent neurospheres are identical, or whether they have species specific characteristics or differences based on their region of origin. Under our culture conditions, rodent neurospheres isolated from the cortex (^{ctx}NS) and striatum (^{str}NS) grew faster than those from the mesencephalon (^{mes}NS), but stopped growing after only eight to ten population doublings. In contrast, human neurospheres under identical culture conditions, continued to grow for over 40 population doublings. Following migration and differentiation of both rodent and human cultures, ^{ctx}NS and ^{str}NS generated high numbers of small neurons whereas ^{mes}NS generated small numbers of large neurons with many long fibres. Only very rare neurons from ^{mes}NS expressed dopaminergic markers, and thus may require further signals to fully mature. While the rat neurospheres generated high numbers of oligodendrocytes, very few were found to develop from human neurospheres from any region after a few weeks of passaging. FACS analysis revealed a unique population of smaller cells within human ^{str}NS and ^{ctx}NS, which appeared to be neuronal progenitors. However, large cells within neurospheres were capable of generating these small neuronal progenitors following further proliferation. Together, our data show that rat and human neurospheres have unique characteristics with regard to growth and differentiation, and that the majority of precursor cells within neurospheres are regionally specified to generate set numbers of neurons. These findings have important implications for understanding the nature of proliferating neural precursors isolated from the developing CNS, and their potential for brain repair.

1. Introduction

Small populations of stem cells exist in the developing and adult rodent brain, which can generate progenitor cells capable of differentiating into neurons, astrocytes or oligodendrocytes [17]. However, fundamental challenges within this field of biology are (i) to establish how cell-autonomous programs interact with environmental signals to direct the phenotypic fate of these cells and (ii) to understand the mechanisms underlying their self-renewal capacity [2]. One technique for growing cells derived from the germinal zones of either the developing or adult CNS involves the generation of free floating spherical aggregates termed 'neurospheres'. This method was developed for rodent tissues a number of years ago [32] and has recently been adapted for the long-term growth of human neurospheres by ourselves and others [10,45,49]. Neurospheres from rodents consist of both multipotent stem cells and more restricted progenitors [33] and, as such, are considered to comprise a heterogeneous population of neural precursor cells (NPCs) [41]. Although the selection of sphere forming cells from primary neurogenic zones within differentiating CNS regions of the animal is possible [34,47], the reliable distinction between true stem cells and more restricted progenitors within expanded populations of neurospheres has been limited by the lack of available cell-type specific markers. It is possible that such cells may be regionally specified. If this were true, neurospheres generated from different brain regions would retain some features of this region, even following expansion in culture. Alternatively, is it possible that a common

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stem cell exists along the entire extent of the neuroaxis which, following isolation, would behave in a similar fashion irrespective of its origin. In addition to these regional complexities, there are likely to be differences between species with regard to general stem cell biology that need to be addressed, particularly in the context of potential clinical applications.

Clearly, tissues derived from one brain region and expanded in culture can take on the phenotype of another following transplantation [16,36,40] or, in more extreme cases, can trans-differentiate into cells of different dermal origin when injected into irradiated mice or blastocysts [5,12]. These results suggest that at least some neuroepithelial cells are extremely plastic and environmentally specified, with very little evidence of genetic determination. However, these types of cells may be only a very small fraction of cells within such cultures. Although cells derived from different embryonic brain regions and expanded in culture adopt host region phenotypes when transplanted, there is evidence that some of these cells retain a molecular memory of where they came from, based on the expression of regionally expressed genes [28,35,53] or proteins [14]. In addition, although cells derived from the embryonic forebrain migrate and differentiate within hind-brain regions when transplanted into neonates [9], these cells continue to express markers associated with their region of origin [27]. Very recently, the region specific differentiation of spinal cord progenitor cells [52] and mammalian neural crest cells [51] has also been reported following transplantation. Thus, cell autonomous mechanisms may exist to control the fate of these cells. Neurospheres generated from the human fetal brain produce large number of neurons [42], but those from the spinal cord exclusively produce astrocytes using slightly different growth conditions [3,31], suggesting that some regional specification also exists along the human neuroaxis. However, a direct comparison of proliferative and phenotypic potential of neurospheres generated from different brain regions and grown under identical culture conditions, has not previously been undertaken.

To address these issues, we compared the growth and differentiation of non-genetically modified epidermal growth factor (EGF)- and fibroblast growth factor-2 (FGF-2)-responsive neural precursors isolated from various regions of the embryonic rat and human brain.

2. Materials and methods

2.1. Rodent neural precursor cell cultures and proliferation studies

The cortex, striatum (comprising both medial and lateral ganglionic eminences) and ventral mesencephalon (VM) were dissected from embryonic day 14 (E14) rat brain. Human embryonic tissue (between 6 and 21 weeks post conception) was collected following routine terminations of pregnancy. The methods of human tissue collection conformed with the arrangements recommended by the Polkinghorne Committee for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health. The same regions plus the cerebellum and thalamus were isolated from human fetal samples. Tissue was treated with trypsin (0.1% for 20 min), washed in DMEM and then dissociated into a single cell suspension. Cells were initially seeded at a density of 400,000 per ml into T75 flasks containing 20 ml of defined serum-free medium (DMEM:HAMS-F12 at 3:1) supplemented with B27 (2% v/v), epidermal growth factor (EGF, 20 ng per ml) and fibroblast growth factor (FGF-2, 20 ng per ml) with heparin (5 μg per ml).

Cells from all regions of both the rat and human tissue formed neurosphere cultures during the first 2–5 days of growth. Due to differences in expansion rates between the rat and human cells, they were then passaged differently from this point. The rat cultures were passaged at 7 days (P1) and then every 14 days (P2, P3) by chopping spheres into 200-μm sections, which were then re-seeded into fresh growth medium containing both EGF and FGF-2 and B27 at a density equivalent to ~200,000 cells per ml. The sectioning of neurospheres has previously been developed as a method for optimising the in vitro expansion of human NPCs [45]. Estimates of rat cell expansion were undertaken every 7 days by the removal of aliquots of cells from the flasks, which were then dissociated and counted using the trypan blue exclusion method. To establish a relationship between cell number and neurosphere volume, sequential measurements of sphere size were undertaken for individual rat neurospheres between 7 and 16 days in culture.

The human neurospheres were passaged every 14 days by sectioning of spheres into 350-μm sections, that were re-seeded into fresh growth medium at a density equivalent to 200,000 cells per ml. Half the growth medium was replenished every 4th day. Passaging of cells was undertaken every 14 days. After the first passage all cells were grown in EGF and FGF-2 supplemented media, but B27 was replaced with the supplement N2 (1% v/v, Gibco). At 4 weeks of growth all cultures were switched to EGF alone and fed every 4 days and passaged every 14 days thereafter. These slight differences to the rodent cultures were introduced because the human cultures could be expanded for long periods of time without the addition of either B27 or FGF-2 and these factors were not required for continual growth of the human cells for up to 250 days [45].

2.2. In vitro differentiation studies and neuronal quantification

We used a cell migration assay, which has been previously described in detail [7], to assess the differentiation potential of neurospheres grown from either the rat or

human. Whole neurospheres generated from the different CNS regions and at sequential passages were plated directly onto poly-L-lysine/laminin-coated glass coverslips in serum-free medium (DMEM:HAMS F-12) containing B27 supplement (2% v/v) but without mitogens. Over a 7–14-day period following plating, cells migrated away from the sphere and formed a differentiating neuronal and glial monolayer. The cells were fixed on day 7 or 14 in 4% paraformaldehyde and rinsed in PBS. Fixed cultures were blocked in 3% goat serum with 0.3% Triton X-100 and incubated with primary antibodies to β-tubulin III (monoclonal, 1:500, Sigma), glial fibrillary acidic protein (GFAP; polyclonal, 1:1000, DAKO), Gal-C (monoclonal, 1:300, Sigma) or tyrosine hydroxylase (TH; monoclonal; 1:500, Chemicon). Following rinsing in PBS, the cultures were incubated in either biotinylated goat anti-mouse or fluorescein-conjugated goat anti-rabbit antibodies. Biotinylated cultures were visualized using a streptavidin-rhodamine conjugate, and Hoechst 33258 was used as a nuclear stain. In order to demonstrate that differentiated cells had arisen from dividing NPCs, some neurospheres were pulsed with BrdU (0.1 μM) for 12 h prior to plating and differentiation. Following fixation, these cells were then co-stained for BrdU following the protocol supplied by a commercially available kit (Boehringer) and either β-tubulin-III, GFAP or Gal-C.

Quantification of cells migrating out from neurospheres was achieved by viewing cells under a fluorescence microscope (×40 objective) and counting Hoechst-stained nuclei along with labelled neurons in at least four independent fields (total area >0.25 mm^2) immediately adjacent to plated spheres using a pre-defined template. Measurements of neuronal cell body areas were undertaken using Openlab 2.1 digital imaging software.

2.3. [^{3}H]Thymidine incorporation assay

Single spheres were exposed to 0.5 μCi per ml of [^{3}H]thymidine for 24 h at 37°C, in the presence of growth factors as appropriate. At the end of the incubation period spheres were washed three times with DMEM and incubated for 30 min at 4°C with 10% trichloroacetic acid (TCA) to remove free [^{3}H]thymidine. The spheres were then rinsed three times with 10% TCA and washed once with 95% ethanol. The incorporated [^{3}H]thymidine was solubilized with 0.5 M NaOH for 30 min at 37°C, which was then neutralized by addition of 1 M HCl. This solution was added to 4 ml of scintillation cocktail and counted in a scintillation spectrometer.

2.4. FACS analysis

Whole neurospheres, or neurospheres plated for 7 days, were incubated in 0.1% trypsin for 20 min, washed in DMEM and then seeded into L15 medium (Gibco) supplemented with B27 (1:50) and kept at 4°C. The cells were then incubated in propidium iodide (PI) for 10 min to label dead cells and then filtered through a sterile high pass filter into a FACS analysis tube. The analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser emission wavelength of 488 nm as described in detail previously [25]. PI and autofluorescence was identified using a 585-nm band pass filter.

2.5. Statistical analysis

All data are expressed as means±S.E.M. and were analysed using two-way ANOVA with Bonferonni post hoc comparisons (GraphPad Prism software version 3.00).

3. Results

3.1. Regional differences in growth rates of rat neurospheres

Cells derived from either the E14 rat cortex (^{ctx}NS) or striatum (^{str}NS) grew as neurospheres following plating and showed exponential growth over the first 35 days (Fig. 1A). These numbers represent a nearly 170-fold expansion in cell number, theoretically equivalent to approximately eight population doublings. In contrast to the forebrain neurospheres, those derived from the mesencephalon (^{mes}NS) underwent an approximate three- to four-fold expansion (from 4×10^6 to $15.2\pm3.2\times10^6$) over the same period, and there was no further increase in the cell number after 21 days in culture (Fig. 1A). Post hoc comparisons between the groups revealed significant differences between effective expansion ratios for neurospheres derived from the forebrain (cortical or striatal) and midbrain ($P<0.05$) at 28 and 35 days in culture. We have previously shown that in contrast to mouse neurospheres, human and rat neurospheres could not be expanded for more than 5 weeks in culture [43,44]. We were able to overcome this growth limitation in human neurospheres by developing a chopping method of passaging which maintained cell–cell contact and allowed extended growth of human neurospheres for up to 150 days [45]. However, the same chopping method did not extend the growth of rat neurospheres. All of the rat neurospheres, regardless of regional origin, underwent senescence at 5 weeks (Fig. 1A). These results suggest that there are fundamental differences between rat and human neurospheres with regard to their continual propagation in culture using these methods.

The regional differences in rat NPC growth rates were further reflected in the relative changes in sphere size measured between day 7 and day 16 of culture (Fig. 1B). Between these time-points, the mean neurosphere diameter increased from 0.33±0.03 to 0.71±0.06 mm for ^{ctx}NS,

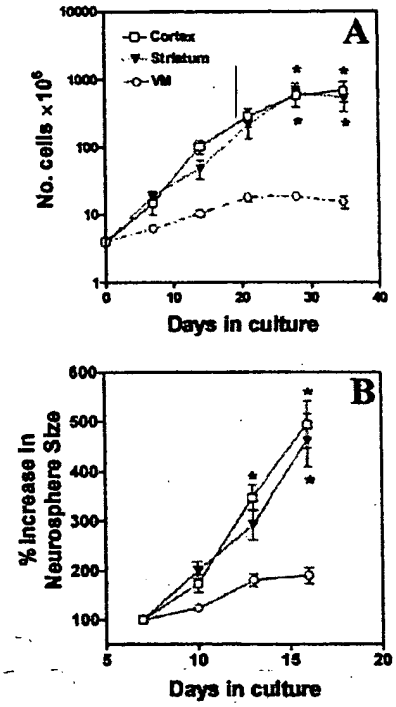

Fig. 1. Cells from the mesencephalon grow more slowly than those from the striatum or cortex. (A) Cell numbers at sequential passages. Data are means±S.E.M. for three independent experiments. Two-way ANOVA revealed a significant overall interaction between the regions across time in culture ($P<0.001$). Asterisks indicate significant difference in cell numbers between forebrain (cortical or striatal) groups and VM group at 28 and 35 days in vitro ($P<0.05$). (B) Neurosphere size expressed as percentage increase in sphere area between days 7 and 16 in culture. Data are means±S.E.M. for $n=12$ spheres per region. Asterisks indicate significant difference between forebrain (cortex or striatal)-derived neurospheres and VM neurospheres ($P<0.05$).

from 0.32±0.01 to 0.65±0.05 mm for ^{str}NS and from 0.26±0.01 to 0.35±0.03 mm for ^{mes}NS ($n=12$ spheres per region). The corresponding percentage increases in sphere size over a 10-day period also revealed significant differences between ^{ctx}NS/^{str}NS and ^{mes}NS ($P<0.05$).

3.2. Regional differences in neuronal production for rat neurospheres

Following mitogen withdrawal, whole neurospheres derived from the respective regions of the embryonic brain, and taken at sequential passages, were allowed to differentiate using an established cell migration assay [7]. In the migration assay, individual cells are required to migrate from the plated neurosphere and onto the substrate in order to be analysed. Therefore, the relative proportions of neurons, astrocytes and oligodendrocytes represent those cells that have actively migrated as progenitor cells out onto the substrate and subsequently undergone differentiation, rather than any remaining post migratory cells. After 7 days in vitro, cells that had migrated out from the neurosphere were seen to express β-tubulin-III, GFAP and Gal-C. In BrdU pulse-chase studies, many of the differentiated neural phenotypes could be co-labelled for BrdU suggesting that they had arisen de novo from the proliferating NPCs (Fig. 2). ^{Str}NS gave rise to significantly more neurons than ^{ctx}NS or ^{mes}NS at 21 days of expansion ($P<0.001$) (Fig. 3A,B). Overall, within each region, there was a trend towards a reduction in neuronal emergence with time (passage) in culture, although this result was not statistically significant. At late passages the ^{mes}NS produced very few neurons. The differences between regions for neuronal differentiation were sustained at sequential time-points in culture (Fig. 3A,B), suggesting that any regionally-defined determinants for neuronal differentiation were conserved despite on-going proliferation ex vivo. All regions produced ~10–20% oligodendrocytes at each passage, although this was not quantified in detail.

In order to determine whether migrating neurons showed distinct region specific morphologies, we assessed cell body area. Neuronal cell body areas were found to be significantly greater for neurons generated from ^{mes}NS than for neurons derived from forebrain neurospheres at 7

Fig. 2. Fluorescent photomicrographs showing differentiated neural phenotypes (red) emerging from whole neurospheres plated onto PLL/laminin under serum-free conditions: β-tubulin III-positive neurons (a), Gal-C-positive oligodendrocytes (b) and GFAP-positive astrocytes (c). Neurospheres were pre-pulsed with BrdU (0.1 μM for 12 h prior to plating). Cells were fixed at 7 days following plating. Newborn cells arising from proliferating neural precursor cells are double labelled for BrdU (green, arrows). Scale bar represents 50 μm.

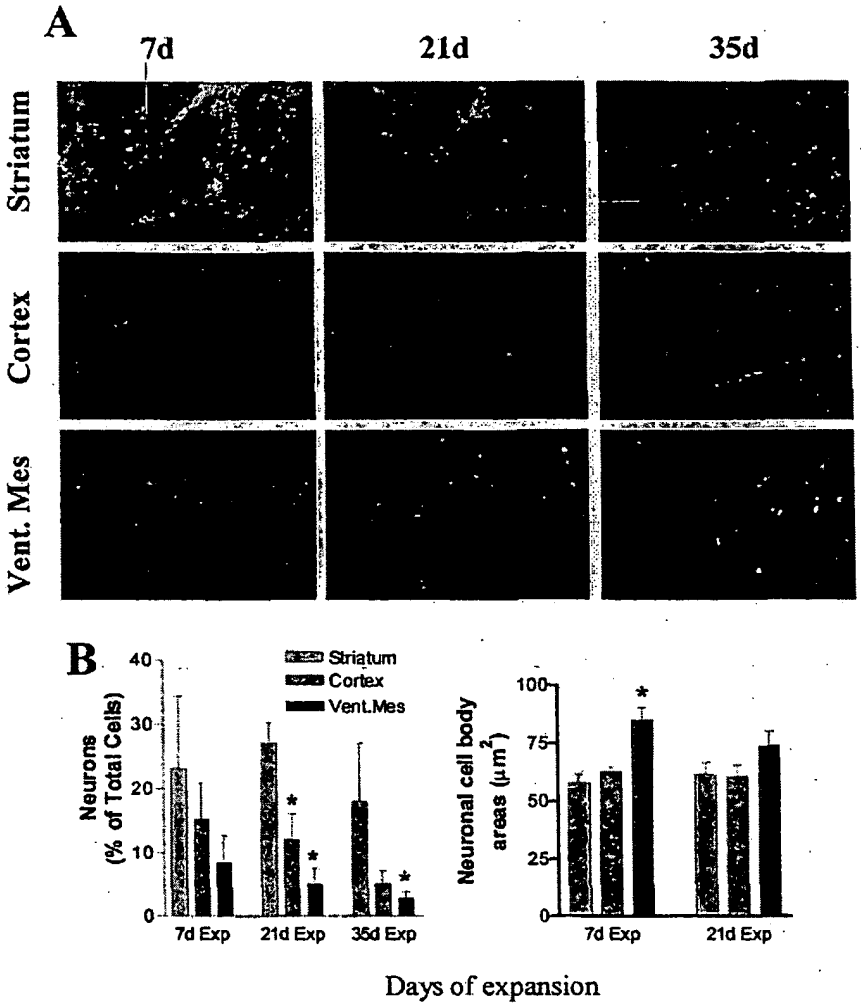

Fig. 3. Rat neurospheres are regionally specified. (A) Following differentiation at each time point, the number and phenotype of cells which had migrated out from spheres were significantly different between the striatum, cortex and mesencephalon. While the ^{str}NS generated large numbers of neurons, both the ^{ctx}NS and ^{mes}NS generated significantly fewer neurons at 21days, and the ^{mes}NS generated fewer neurons at 35 days (star, significantly different from cortex at $P<0.001$). (B) There were significantly larger cell bodies for β-tubulin III-positive neurons derived from expanded VM neural precursors when compared to neurons derived from forebrain (either cortical or striatal) precursors (asterisk indicates significance at P0, $P<0.05$). A trend for this difference was maintained at 35 days (P2). Data represent means±S.E.M. across three separate experiments.

days in vitro ($P<0.05$), and a trend for this difference was retained even after 21 days in vitro (Fig. 3B). We next assessed whether there were any specific phenotypic markers retained by the VM generated neurospheres. In particular we examined for tyrosine hydroxylase expression, which might indicate dopaminergic differentiation amongst proliferating NPCs. In keeping with our previous report [8] only a small number of well-developed TH-positive neurons were seen when neurospheres were plated following 14 days of growth. Moreover, these cells were found to reside within the limits of the neurosphere and possibly represented primary dopamine neurons which had not subsequently divided in culture (data not shown). At later passages, it was not possible to identify any TH-immunoreactive neurons in neurospheres generated from any region.

3.3. Regional specification of human neurospheres

We have previously shown that neurospheres derived from the human embryonic forebrain can be grown in culture for extended periods of time providing that cell–cell contact is maintained [45]. In the current study, human tissue from all brain regions also produced spheres after

dissociation and seeding into EGF and FGF-2 supplemented culture medium (Fig. 4A). These neurospheres continued to increase in size over time and were passaged using the chopping method. In contrast to the rat neurospheres, human neurospheres derived from all regions showed continual growth over the first 20 weeks of culture, although this was slower than that seen for the rat neurospheres. After 20 weeks of growth there were no significant differences in [^{3}H]thymidine uptake (a measure of cell proliferation) in neurospheres generated from the different brain regions (Fig. 4B). However, there was a consistent trend for the ^{mes}NS to grow at a slower rate than those from other regions. Upon mitogen removal and exposure to 1% serum and laminin, the spheres attached and cells rapidly migrated out onto the substrate. Clear differences in the total number of cells migrating were apparent in fields around neurospheres generated from each region (Fig. 4C). This was in large part due to the high proportion of small, phase bright immature neurons emerging from the ^{ctx}NS and ^{str}NS, which labelled with the neuronal marker TuJ1 (Fig. 4D,E).

Detailed analysis of the neuronal morphology revealed that cortical neurospheres gave rise to neurons that were significantly smaller than those from mesencephalic neurospheres (mean area of cortex-derived neurons±S.E.M.= 64.5±1.59, n=154 cells; mesencephalon-derived neurons=137.6±6.00, n=67 cells: significantly different at $P<0.0001$, Student's t-test). Furthermore, neurons from mesencephalic and cerebellar neurospheres often had long axonal process with characteristic blebs, not seen in neurons from cortical or striatal neurospheres (Fig. 4D, arrowheads), although very few cells from any region (<0.01%) stained for tyrosine hydroxylase. Neurons from striatal neurospheres did not stain for choline acetyltransferase or dopamine and adenosine-related phosphoprotein (DARPP-32), but many were positive for GABA and glutamate as described previously [7] suggesting only selective neurochemical phenotypes were emerging from these neurospheres. We have recently shown that addition of growth factors further increases the number of cortically derived neurons [7]. In contrast, the small number of neurons generated from hind-brain neurospheres did not

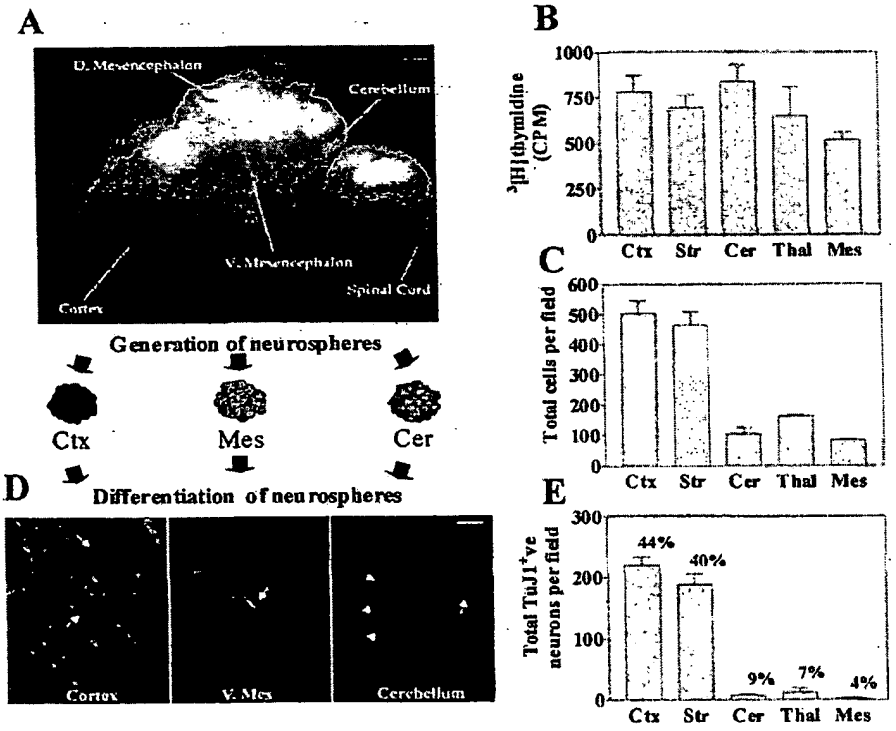

Fig. 4. Human neural precursor cells are regionally specified. (A) Brain tissue was removed from post mortem human fetal tissues (8–10 weeks post conception). (B) [^{3}H]Thymidine uptake over 24 h by neurospheres derived from the cortex (ctx), striatum (str), cerebellum (cer), thalamus (thal) or mesencephalon (mes) showed similar rates of uptake, with the lowest being the ^{mes}NS. (C) Total number of cells which migrated out from each region over 14 days. Note the marked differences in total migrating cells between the cortex and striatum compared with other regions. (D) Staining of the migrating cells from the different neurospheres with antibodies to TuJ1 (red; neuronal marker) and GFAP (green; astrocyte marker) revealed small neurons without long axonal processes from the striatum and cortex, but larger neurons with longer processes from other brain regions.. Nuclei were labelled with Hoechst (blue). (E) The cortex and striatum were unique in generating large numbers of neurons. n=3–5 separate foetal samples. Scale bar represents 50 μm.

significantly increase upon growth factor addition, suggesting that trophic factor dependent cell death is not responsible for the differences in neuronal number (data not shown). In contrast to the rodent cultures described above, only a small number of oligodendrocytes were generated from early passage human neurospheres from every region used in this study (~1%), and the number of oligodendrocytes further declined to very low levels at later passages (<0.01%).

To determine how much cell division occurs following plating, and to establish the types of cells from ^{ctx}NS that give rise to the small Tuj1 positive neurons, we used time lapse cinematography to image the cells as they emerged from the sphere. At 3 days after plating a number of cells were seen to be dividing within the region around the sphere in the absence of either EGF or FGF-2. These cells were either very small, oval migratory cells or large more static cells with a type 1 astrocyte morphology. All of the divisions within this 2D environment were symmetrical (Fig. 5; see website for live image: http://www.waisman.wisc.edu). A large cell was never seen to give rise to a small cell and large cell upon division under these conditions (over 200 divisions followed for 24 h). Thus, the large number of neurons that emerge from the ^{str}NS or ^{ctx}NS could in part be due to division of small migratory neuronal progenitors post-plating, as described previously in the rodent system [23].

3.4. FACS analysis confirms regional differences within growing spheres

We next wanted to establish the nature of the dividing cells within the human neurospheres. Since we were unable to clone single cells from these cultures (they require continual cell–cell contact to divide under the present culture conditions), and our time lapse data did not show asymmetric divisions, we were not able to prove that a single precursor was dividing asymmetrically to generate both neurons and astrocytes. It was possible that two cells were dividing alongside each other: one a small neuronal progenitor (enriched in cortical primary tissue) and the other a large astrocyte progenitor (enriched in hind-brain primary tissue). To investigate this further, a method previously developed to distinguish various cell types within rodent neurospheres based on flow cytometry (FACS) was used [25]. FACS analysis of human neurospheres based on cell size and auto fluorescence revealed that there was a population of small, weakly fluorescent cells found only in neurospheres derived from the forebrain (Fig. 6A, R3). Following differentiation for 7 days, the proportion of small cells increased dramatically in forebrain neurospheres (Fig. 6B, R3). In addition to the increase in this small cell population, the cells themselves were apparently smaller. Similar populations of small cells were absent from the ^{mes}NS (Fig. 6B, R3). These results

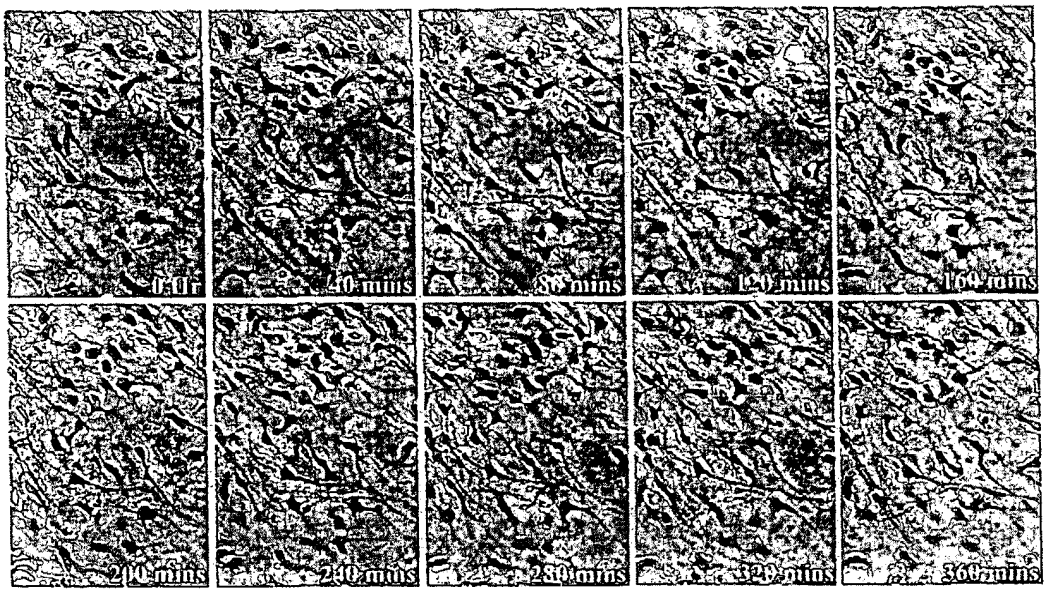

Fig. 5. Time lapse recording of cells around a human neurosphere dividing and migrating. Spheres were plated onto laminin in the absence of EGF and allowed to differentiate for 3 days. Interestingly, even in the absence of mitogens some division continued post plating. (A) Representation of a small migratory neuroblast which would invariably be TuJ1 positive (Fig. 7). (B) Representation of a small migrating neuroblast which underwent cell division (arrows) to give rise to two anatomically similar daughters which then migrated away. (C1 and C2) Representation of two large cells with an astrocytic morphology which underwent cell division (arrows) and gave rise to very similar large daughter cells. These large cells were far less migratory than the smaller ones. A video sequence can be seen in the on-line version of the paper, available on http://www.neuroscion.com.

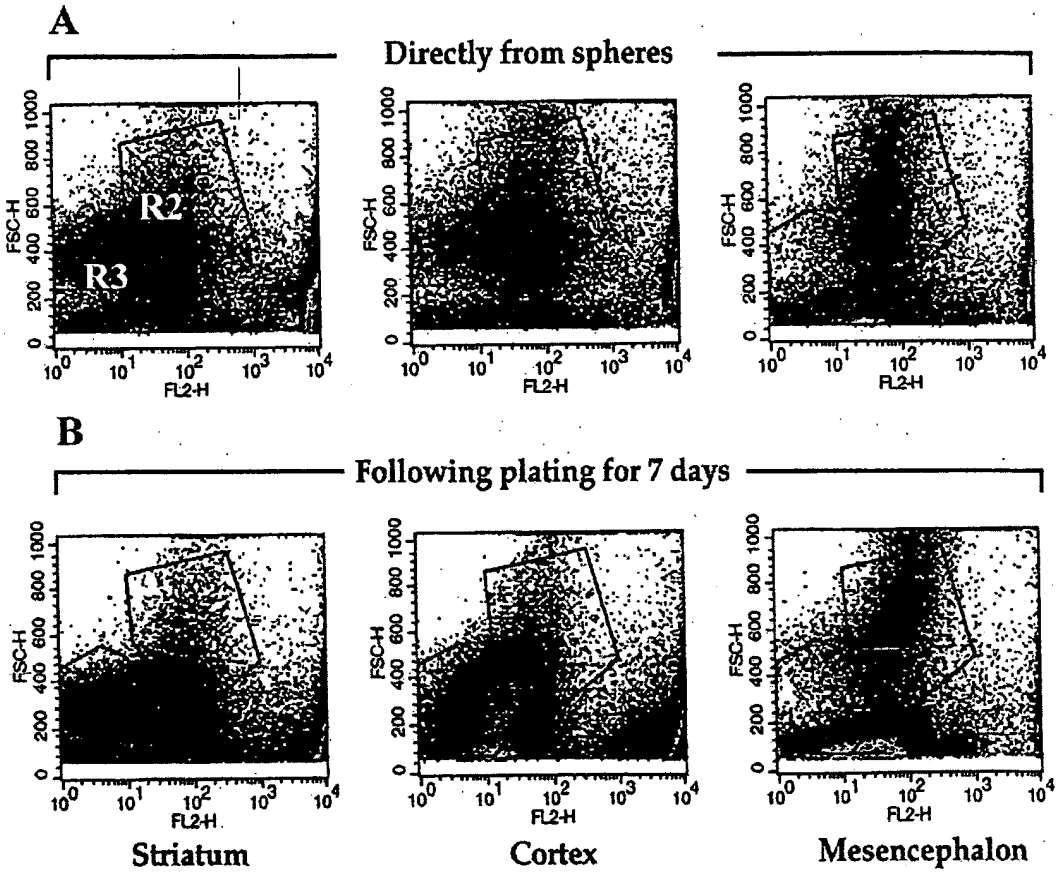

Fig. 6. FACS analysis confirms regional differences are apparent even within neurospheres. (A) Human neurospheres from different brain regions grown for 20 weeks as described in the Materials and methods section were dissociated and stained with propidium iodide, which labels dead cells, and sorted by size (*Y*-axis) and intensity of fluorescence (*X*-axis). Dead cells appear as highly fluorescent band to right of plot. Each dot represents a single cell. Neurospheres from the cortex and striatum showed a side band of smaller, less fluorescent cells (in region R3) which were missing in neurospheres from the mesencephalon. (B) Following differentiation for 7 days, the ^{ctx}NS and ^{mes}NS gave rise to many small cells which appear in R3. These were not seen to develop from the mesencephalon derived neurospheres. This analysis was repeated using three separate cultures with similar results.

therefore suggest that these cells represent the large number of small neuronal progenitors seen to arise from these cultures, and shown to be actively migrating in Fig. 5.

To determine whether the unique population of small cells in neurospheres derived from forebrain were indeed small self-renewing neuronal progenitors, cells from human ^{ctx}NS were sorted and separated in to large (R2) and small (R3) populations (Fig. 7A). These cells were either plated onto laminin to assess differentiation, or seeded at high density with EGF to generate new neurospheres. After plating onto laminin, R2 cells showed a large amorphous phenotype, and a number of these had already begun to express GFAP after only 24 h (Fig. 7B). By 7 days nearly all of the cells in these cultures were GFAP positive (not shown). No Tuj1$^+$ve neurons were detected in these cultures at any time point. In contrast, after plating onto laminin the small R3 cells were enriched for TuJ1$^+$ve neurons while still containing a few GFAP$^+$ve astrocytes (Fig. 7B).

Did the large cells, which form mostly astrocytes and no neurons following plating onto laminin (presumably due to not undergoing asymmetric divisions), have the potential to generate neurons? To test this we attempted to make new neurospheres from the sorted cell populations. Following re-exposure to EGF immediately following plating onto non-coated plastic dishes, the small R3 cells failed to generate any new neurospheres (Fig. 7C). In contrast, the large R2 cells plated at high density in the presence of EGF rapidly aggregated and formed new spheres (Fig. 7C). Following 14 days of growth, intact spheres derived from the large R2 group were re-plated as whole spheres onto laminin and allowed to differentiate. Within 7 days they had generated large numbers of small TuJ1$^+$ve

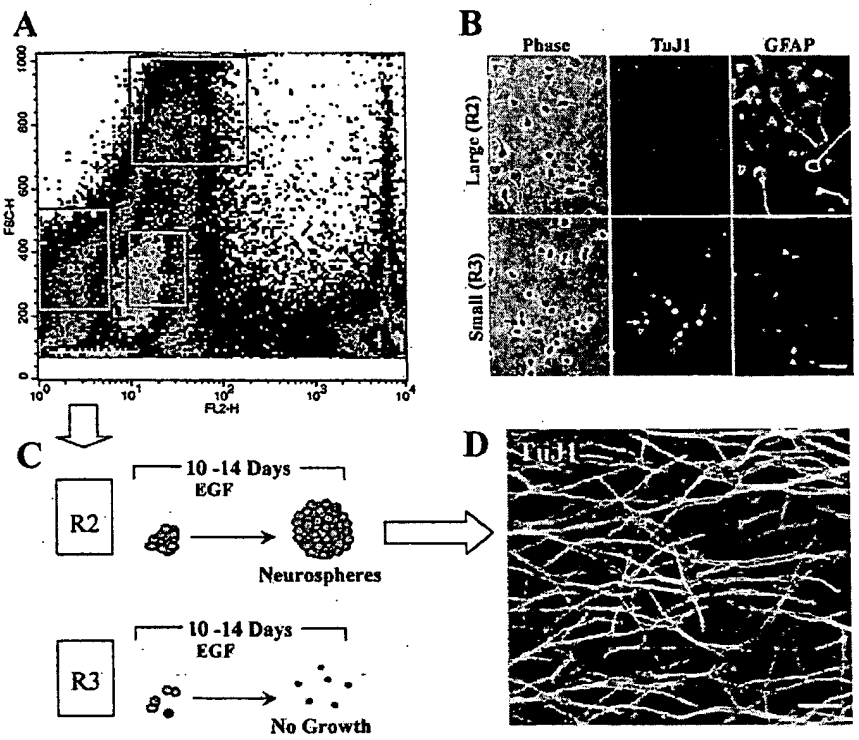

Fig. 7. A large cell with a glial morphology can give rise to neurons. (A) Neurospheres generated from the human cortex and grown for 20 weeks were sorted by FACS and two regions collected (large R2 and small R3). (B) Following acute plating (2 h) onto coated wells, the small R3 cells gave rise to a high numbers of Tu1 positive neuroblasts (arrows) with a few GFAP positive cells (arrowheads). In contrast the large R2 group gave rise to both GFAP (arrowheads) and unlabelled cells but no Tuj1 positive neuroblasts. Scale bar represents 80 μm. (C) Following re-exposure to EGF at high plating densities the R2 group generated new neurospheres whereas the R3 group did not respond to EGF. (D) Following 2 weeks of expansion, neurospheres generated from the R3 group again generated large numbers of small Tuj1 positive neurons following plating for 7 days. Scale bar represents 50 μm. This analysis was repeated twice with two separate cultures with similar results.

neurons (Fig. 7D). This demonstrates that the large cell population within the ^{ctx}NS is the neurosphere forming cell. Furthermore, this cell is able to generate a new population of small neuronal progenitors within the 3D environment of the neurospheres.

3.5. Discussion

3.5.1. Limited growth of rat but not human neurospheres

In this study, and in our previous report [44], we were unable to grow rat ^{str}NS for more than 6 weeks. Various techniques shown to enhance the growth of rat neurospheres, such as maintaining cell–cell contact, adding heparin to the medium, combining EGF and FGF or adding LIF to the medium (reviewed in Ref. [41]) did not overcome this lack of growth in the current study. The growth restriction was not simply a function of embryonic age, as a similar cessation in growth occurs in neurospheres derived from tissue between E12 and P1 (Rossor and Svendsen, unpublished observations). Neurospheres generated from other brain regions also stopped growing after a short period of expansion. Paradoxically, rodent neurospheres express high levels of telomerase and have long telomeres [29], suggesting that they have enormous potential for growth. Human neurospheres, on the other hand, express only small amounts of telomerase and have shorter telomeres, yet grow for much longer periods [29]. Clearly in neurospheres derived from different species, there are cell proliferation checkpoint mechanisms that are independent of telomerase activity. Furthermore, it is likely that the culture conditions for rat neurosphere growth have not yet been optimised, and that their early cessation of proliferation could be overcome in the future. Indeed, rodent oligodendrocyte precursor cells, which normally can only undergo eight divisions in culture, can continue to divide for extended periods of time providing thyroid hormone is removed from the media [46]. We look forward to future improvements in the growth of rat neurospheres as well.

3.5.2. Regional specification of neurospheres in the absence of environmental signals

Classical retroviral studies have shown that at early stages of development, at least some single labelled cells can give rise to multiple types of neuron and glia [37]. Thus, there is widespread agreement that true multipotent neural stem cells exist in vivo. However, similar studies have also shown that within the developing cortex, many cells are more restricted progenitors, such as those giving rise to either pyramidal or non-pyramidal neurons [30]. Furthermore, cortical and striatal progenitors retain the capacity to differentiate into specific phenotypes, even when removed from their in vivo environment and induced to divide several times in vitro [14,38]. The fate of cortical neurons appears to be critically dependent on the signals the cells receive in the final stages of cell division [24]. In the neurosphere model used in the present study, the environmental cues are limited to the surrounding cells, all undergoing synchronous differentiation. These surroundings may be very different to the in vivo situation where polarity, growth factor gradients and a temporally defined range of different cells are influencing cell fate. Under the culture conditions employed here, all neurospheres gave rise to differentiating neurons, although the number and morphology were very different. Since at least some of these neurons had arisen from dividing neural precursors, as indicated by BrdU co-labelling, the regional differences in neuronal emergence are most unlikely to reflect differences amongst post-migratory neurons remaining from the primary culture. Rather, they are likely to reflect the presence of regionally-specified cell-autonomous signals retained within neurospheres.

The migrating neuroblasts from the ^{mes}NS were consistently larger than those from the ^{str}NS or ^{ctx}NS and often had long axonal projection fibres. We propose that in the absence of other signals, there must be a program within the dividing and migrating progenitor cells which determines cell size and differentiation. As such they might be considered lineage restricted, perhaps in a similar fashion to the long-term and transiently self renewing populations of hematopoietic stem cells described previously [26]. Alternatively, the surrounding migrating glial cells may release factors that affect the size and number of migrating neuroblasts. It of course remains possible that under different culture conditions, or following grafting, the fate of these progenitor cells may be changed, i.e. they are not determined, but specified in the absence of other signals. In addition, there may be a few true multipotent, or even pluripotent stem cells in neurospheres from each brain region dividing slowly alongside more rapidly proliferating restricted neural progenitors (Fig. 8). These would be missed when large numbers of cells are plated simul-

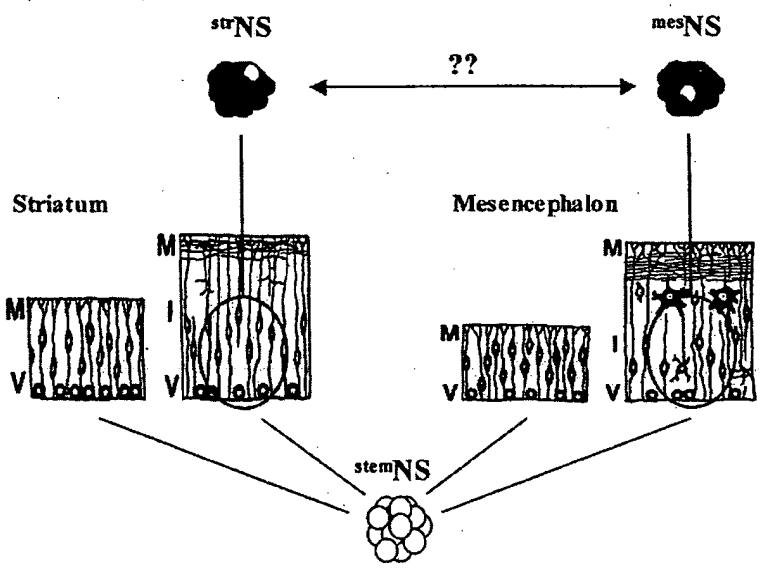

Fig. 8. Neurospheres are regionally specified. Our initial prediction was that perhaps a common cell could be isolated from all regions of the developing brain and grown into a neurosphere (^{stem}NS in white). Instead, the majority of cells within the spheres had regional specificity, even after extended passaging. Those from the striatum (red) had a greater capacity to migrate out from the sphere and form neurons when compared to those from the mesencephalon (green). Our data do not exclude the possibility of non-specified, equipotent stem cells existing within spheres from both regions in very small numbers (white), which exist in ventricular zones (V) throughout the developing neuroaxis. This cell may be masked by the many specified cells. Nor do we know whether under different environmental conditions the fate of spheres from each region might be altered (dotted line). However, we hypothesize that the majority of cells within neurospheres arise from regionally specified progenitors within the intermediate zone (I). Further studies are now required to establish the mechanisms underlying these observations.

taneously. It is possible that these pluripotent stem cells may be responsible for the reported trans-differentiation of human neurosphere derived cells into muscle cells at low efficiency [18].

It is tempting to speculate that the relatively high proportion of neurons arising from rat ^{str}NS reflects the protracted period of neurogenesis in the rat striatum, extending from embryonic day 12 (E12) to E22 with a peak at E15/E16 [15]. In comparison, cortical neurogenesis in the rat occurs during a more restricted 6-day period from E13.5 to E19.5 [4]. Similarly, the neurons of the rat substantia nigra are generated from dividing precursor cells in the ventral mesencephalon over only 4–5 days, between E11 and E15 [1,19,21]. These features of neural development are consistent with the present findings that the ^{str}NS gave rise to more neurons than those from either ^{mes}NS or ^{ctx}NS. Thus, the rat neurosphere culture model may more closely resemble in vivo neural development than had previously been realized. The human neurosphere cultures were also regionally specified, but had a far greater capacity for division. Human neural development takes place over at least 9 months with complex patterns of division and migration for each brain structure. It is possible that the protracted division compared to the rat also represents a greater capacity for human cells to divide in vitro and produce the greater quantity of neurons found in the human brain compared to the rodent. We are currently further studying mechanisms of continual proliferation in these human neurosphere cultures.

It was notable that, despite the novel passaging techniques used here for optimising cell–cell contact between proliferating NPCs, the ^{mes}NS only gave rise to very small numbers of TH-positive neurons in comparison to primary mesencephalon cultures. This is consistent with the data suggesting that embryonic dopamine neuroblasts have a limited capacity to undergo in vitro proliferation and expansion [6,8,39]. Indeed, it is recognised that the effective in vitro expansion and/or induction of the DA phenotype in propagated NPCs is likely to require their genetic modification in combination with the appropriate extrinsic inductive signals [50]. Therefore, while there are clear regional differences in the numbers and size of neurons, the generation of neurochemical phenotypes specific to each region is likely to require additional signals which are not present in our culture system. It is of interest that the human cerebellar and thalamic derived neurospheres also generated low numbers of neurons. Based purely on developmental maturation, the cerebellum might be expected to contain the most primitive type of stem cell, and to retain the capacity to produce high numbers of neurons due to the enormous volume of cerebellar granular cells found in the mature human brain. However, this was not the case. It might be that there is an optimal developmental window for harvesting human neural cells capable of making neurospheres with a high neuronal differentiation potential, and that this may differ between brain regions. We are currently investigating whether this is in fact the case.

3.5.3. The special capacity of forebrain neurospheres to generate high numbers of neurons

Why should the neurospheres derived from the human forebrain produce such high numbers of small neurons even after extensive passaging, when the mesencephalon, thalamus and cerebellum produced relatively few? The sub-ventricular zone (SVZ) of the adult rodent forebrain has been shown to harbour cells expressing astrocyte markers (type B cells) which are capable of producing neuronal progenitors (type A cells) via an intermediate cell (type C cell [13]). The type A neuronal progenitors then migrate along the rostral migratory pathway into the olfactory bulb [22]. This three cell system, where the type C cell is capable of generating large numbers of small migrating neurons, is not only unique to the mammalian forebrain, but is also absent in hindbrain structures which do not have an SVZ or in avian species. A very similar three cell system has recently been described in the primate forebrain [20]. We have shown through FACS analysis that a large cell within the forebrain neurospheres, which upon acute differentiation and plating exclusively generated astrocytes, was able to generate neurons when re-exposed to EGF and expanded again in neurosphere cultures. It is possible that this cell may be a type B cell, which within the three dimensional environment of the neurosphere is able to generate type C cells that in turn produce small type A neuronal progenitors. This cell may not be present in neurospheres derived from other brain regions, which were only able to generate neurons directly from the type B cells with no type C intermediate. Although this hypothesis remains highly speculative, it is worthy of further investigation and provides at least one potential mechanism by which the cortical and striatal human cultures are able to maintain such a high neuronal output.

We show here that neurospheres generated from all regions of the human brain were able to produce both astrocytes and neurons, but very few oligodendrocytes at later passages. The lack of oligodendrocyte production seen here and by others at late passages using similar culture systems [10,48] suggests perhaps that either (i) the methods used to grow long-term neurosphere cultures favour committed neuron/astrocyte progenitors rather than multipotent neural stem cells or (ii) they are all multipotent stem cells, but the culture conditions are not correct to produce oligodendrocytes. It is very difficult to distinguish between these two possibilities until we understand more about these cells. However, there is again a clear species difference in that the rodent cultures described produce many oligodendrocytes even if generated from regions of the spinal cord not expected to produce them [11]. A lack of oligodendrocytes does not imply that they could not be

generated from these human cultures under the correct culture conditions, or following transplantation. However, we have so far failed to achieve this effect using a range of different paradigms (Chandran and Svendsen, unpublished observations).

3.5.4. Implications for cell therapy using human neural stem cells

The data presented here suggest that there are significant regional differences when neurospheres are derived from different areas of the developing rodent or human brain. Thus, many of the cells dividing within neurospheres retain a memory of their origin. This may be a result of the different developmental stages the regions were at when cultured, or simply that each neurogenic zone of the developing brain has a specific type of cell which responds to EGF and FGF-2. Regardless of the mechanism underlying this phenomenon, it will be important for transplantation studies using cells derived from human neurospheres to establish their exact origin. It is possible that those from regions other than the striatum or cortex are not likely to generate large numbers of neurons following transplantation, although the effects of different environmental signals now need to be investigated. Hindbrain neurospheres produce less neurons, but those that were generated were larger and had longer processes. Thus we speculate that while cortical/striatal neurospheres may be useful for replacing lost inter-neurons, hindbrain neurospheres may be better suited to replacing large projection neurons. However, this remains to be established in transplantation models.

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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin E. Reubinoff, et al.	**Examiner:**	Deborah Crouch, PhD
Serial No.:	09/970,543	**Art Unit:**	1632
Filed:	4 October 2001	**Confirmation No:**	1839
For:	EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM		

Commissioner for Patents
Alexandria, VA 22313-1450

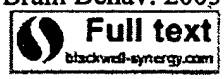
DECLARATION OF CLIVE NIELS SVENDSEN UNDER 37 C.F.R. §1.132

This is the exhibit marked as "Exhibit C" referred to in the Declaration of **CLIVE NIELS SVENDSEN**

By: _____ Dated: 04/03/05.

Exhibit C

Genes Brain Behav. 2003 Jun;2(3):179-86.



Human neural stem cells: a new tool for studying cortical development in Down's syndrome.

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The clinical characteristics of Down's syndrome (DS), or trisomy 21, are caused by errors that occur during development. In addition to mental retardation, DS individuals have craniofacial abnormalities, clinical defects of the heart, gut and immune system, as well as predisposition to certain diseases, such as leukemias and Alzheimer's disease. To explain the developmental mechanisms that cause these traits, it is necessary to look at how developmental processes in DS compare to normal development. The neurological characteristics of DS are established during the prenatal and early postnatal period in humans, when the bulk of brain development occurs. Mouse models of DS have provided a useful way of studying DS neural development. However, there are clearly significant differences between rodent and human biology that may not be reflected in mouse models. Recent advances in stem cell biology now allow the generation of human neural tissue in the culture dish (Ostenfeld & Svendsen 2003). Stem cells offer a novel model system to study alterations in neuron development in developmental disorders such as DS.

PATENTS

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DECLARATION OF CLIVE NIELS SVENDSEN UNDER 37 C.F.R. §1.132

This is the exhibit marked as "Exhibit D" referred to in the Declaration of **CLIVE NIELS SVENDSEN**

By: _____ Dated: 04/19/05.

EXHIBIT D

Brain Pathology 9: 499-513(1999)

SYMPOSIUM: Neural Stem Cells

Human Neural Stem Cells: Isolation, Expansion and Transplantation

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Neural stem cells, with the capacity to self renew and produce the major cell types of the brain, exist in the developing and adult rodent central nervous system (CNS). Their exact function and distribution is currently being assessed, but they represent an interesting cell population, which may be used to study factors important for the differentiation of neurons, astrocytes and oligodendrocytes. Recent evidence suggests that neural stem cells may also exist in both the developing and adult human CNS. These cells can be grown *in vitro* for long periods of time while retaining the potential to differentiate into nervous tissue. Significantly, many neurons can be produced from a limited number of starting cells, raising the possibility of cell replacement therapy for a wide range of neurological disorders. This review summarises this fascinating and growing field of neurobiology, with a particular focus on human tissues.

Introduction

Work on flies, worms and amphibians has provided enormous insight into the molecular mechanisms underlying neural development. This is a result both of the ability to genetically manipulate these animals in large numbers and the accessibility of the developing embryo to observation (18, 34). However, in mammals it is more difficult to assess the intrinsic fate of related neural cells, or the effects of other cells on them. During development, mitotic regions adjacent to the ventricle are composed of neuroepithelial germinal cells which may best be described as neural stem cells (33). These proliferate within the ventricular zone, and give rise to progenitors for both neurons and glia. Many of these migrate and mature into the various regions of the CNS, while a small population may reside within this region as stem cells, possibly into adulthood (for an overview of this process see Figure 1).

Recently, cells have been isolated from the rodent CNS which can divide in culture while retaining the capacity to differentiate into neurons, astrocytes and oligodendrocytes, and as such may represent an "*in vitro*" source of neural stem cells (23, 38, 41, 44, 46, 70, 74, 88). This opens up a new way in which some aspects of neural development can be studied *in vitro*, particularly with regard to the underlying mechanisms controlling the lineage and fate commitment of different groups of mammalian precursor cells. In addition, if neural stem cells can be isolated from human tissues they may represent a rich source for a range of cell therapy programmes aimed at treating neurological disease, many of which are currently dependant on primary fetal tissues (22). But can neural stem cells be isolated from human tissues? How do they compare with their rodent counterparts and how long can they really be propagated in culture? Do they survive transplantation into the adult brain? These are the main topics of this review.

Dividing neural precursor cells in cultures of the embryonic rodent CNS

It was the pioneering work of Harrison at the start of this century, which opened the way to an alternative method of studying the developing nervous system (31). He showed that cells isolated from the embryonic CNS could be maintained outside of the body using specialised nutrient broths. Within these cultures, developing neurites were found to emerge from isolated nerve cells, thus confirming Ramón y Cajal's earlier observations of a growth cone within fixed tissue sections (58). Since these early experiments, cell culture has played a

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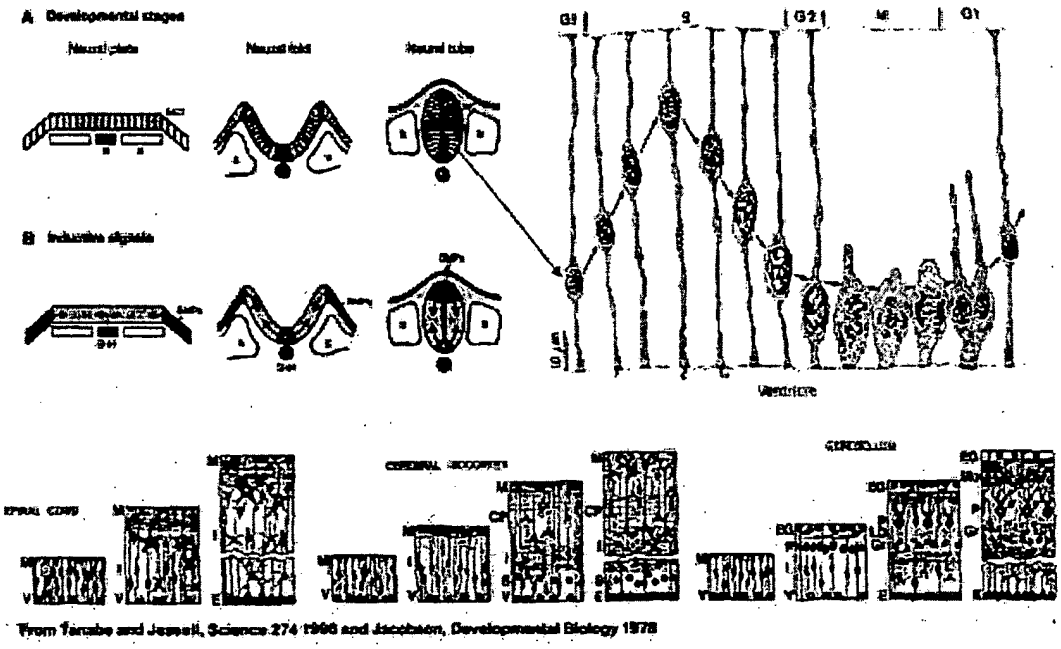


Figure 1. Schematic showing early developmental stages. (A) Exemplified here for the spinal cord, the neural plate folds to produce the neural tube which is lined with dividing neuroepthileal cells and a specialised floor plate region (F) below which lies the notochord (N). (B) Inductive signals from the floor plate (Sonic Hedgehog, shh) and roof plate area (bone morphogenic proteins, BMPs) control some aspects of differentiation (adapted from (83)). Within the neural tube, cell division occurs which is characterised by interkinetic nuclear migration between the ventricular and outer surface, with cell division actually occurring on the ventricular side (68). Through continual division followed by migration, the layers of the CNS are built up over time as shown for the spinal cord, cerebral isocortex and cerebellum. Early to late development are shown from left to right for each region. CP, Cortical plate; E, ependymal layer; EG, external granule layer; Gr, granule layer; I, intermediate zone; LD, lamina dissecans; M, marginal zone; Mo, molecular layer; P, Purkinge cell layer; S, subventricular zone; V, ventricular germinal zone. Reproduced from (33) with permission.

major role in understanding some of the basic mechanisms of neuronal maturation and axon outgrowth. The discovery that fibroblast growth factor-2 (FGF-2) could induce the proliferation of neural precursors within embryonic hippocampal cultures opened up a new avenue of investigation, where proliferation of neural precursors could also be studied *in vitro* (25). Later, a second mitogen, epidermal growth factor (EGF), was found to stimulate the division of embryonic striatal precursor cells which retained the ability to differentiate into neurons, astrocytes and oligodendrocytes (60). In addition, the ventricular zone and hippocampal regions of the adult rodent brain were also found to harbour cells which proliferated in response to FGF-2 and EGF and in many ways appeared similar to their embryonic counterparts (61, 63). Surprisingly, regions of the brain not normally associated with neurogenesis in the adult rodent also contain cells which divide in response to FGF-2, suggesting that quiescent precursor cells may be scattered throughout the entire CNS (49).

Stem cells, progenitors and precursors

There is still much debate over how to classify these germinal cells, particularly when they are removed from the developing or adult CNS and grown *in vitro*. A somewhat liberal approach to labelling a range of dividing cells as "stem cells" has been suggested recently, in an attempt to form a concept of where this very new field of neurobiology currently stands (1). Previous attempts have referred to other tissue systems where stem cells are classically defined as self-renewing, often for the lifetime of the organism, multipotent and able to regenerate damaged tissue (29). Thus, neural stem cells should be able to self-renew, retain the capacity to generate neurons, astrocytes and oligodendrocytes and be capable of replacing these cells when damaged.

Progenitors are similar, but normally restricted to either one or two lineage's and capable of fewer divisions. Precursors are simply the ancestors of subsequent generations and are taken simply to mean dividing cells - they could be either stem cells or progenitors (23). How can these criteria be met in practical terms? The benchmark method for defining a stem cell is clonal analysis where a single cell from a population of cells is plated in isolation and induced to produce daughters through division. These can later be assessed for their potential to generate neurons, astrocytes and oligodendrocytes. This method is enormously powerful when a positive result is seen, i.e. all three phenotypes are generated from a single clone, as one can say conclusively that this particular cell was multipotent and likely to be a stem cell.

Clonal experiments have shown that single cells derived from the embryonic cortex can divide a small number of times in culture and give rise to neurons, astrocytes and oligodendrocytes (17, 55). FGF-2 responsive precursors isolated from the embryonic hippocampus or spinal cord (43) or adult hippocampus (35) and grown as a monolayer culture, have also been shown to be stem cells (50). In some cases, these respond in a specific manner to exogenous factors; platelet-derived growth factor inducing neuronal differentiation and ciliary neurotrophic factor inducing astrocytic differentiation (35). A similar FGF-2 responsive cell, shown to be multipotent using clonal analysis, has been isolated from the adult striatum but this time grown as a free floating suspension or "neurosphere" culture (28). EGF can also drive a multipotent stem cell within neurosphere cultures generated from the embryonic or adult striatum (61, 62), at least at early passages, although the numbers of neurons spontaneously differentiating from these cells is very small (2). This may relate to the fact that under certain circumstances EGF drives a unipotent glial precursor while FGF drives a bipotent neuronal/glial precursor (37). Thus within EGF responsive mouse neurospheres a small number of pluripotent stem cells may be surrounded by other more restricted glial progenitor cell populations.

When a multipotent stem cell has been isolated in culture and cloned, does this mean that large populations of stem cells will be generated from it when it is driven by mitogens? Two situations are possible. In the ideal world, multipotent clones would divide symmetrically in culture, producing an endless supply of new multipotent cells (Figure 2). However, based on *in vivo* data these cells are often likely to divide both symmetrically and asymmetrically (15), leading very rapidly to

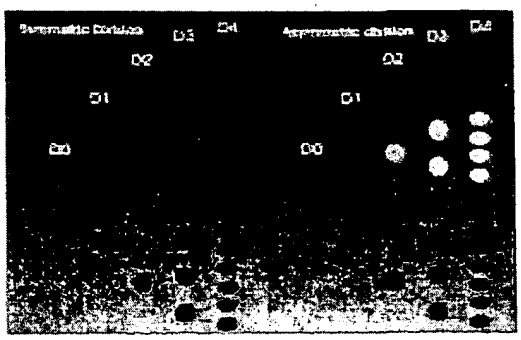
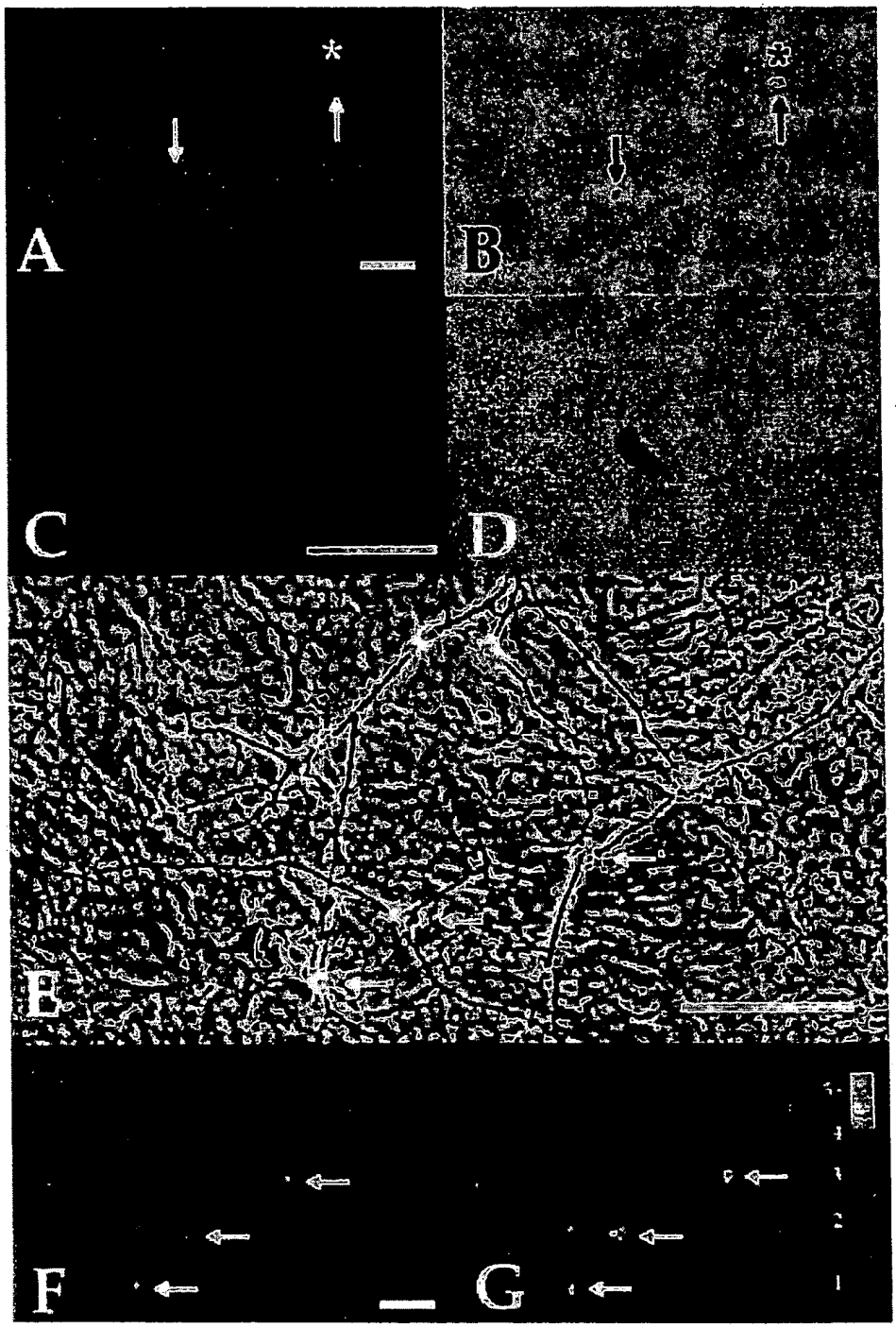


Figure 2. Schematic of the two possible types of cell division. Neural stem cells have to self renew. In symmetric divisions a single stem cell (red) is able to give rise to many other stem cells with identical properties. In reality, dividing cells in culture are more likely to undergo both symmetric and asymmetric divisions, leading to a wide variety of possible phenotypes and a heterogeneous culture after only four population doublings.

a heterogeneous culture containing stem cells, progenitors and even terminally differentiated cells (Figure 2). This will, to a degree, depend on the exact culture conditions, but where large numbers of cells have been propagated *in vitro*, they are likely to be heterogeneous even when arising from a single cell. Evidence for this comes from the detailed analysis of the phenotypic potential of individual clones isolated from adult hippocampal neural stem cells. These showed a large degree of intra-clonal variation with regard to the percentage of neurons, oligodendrocytes and astrocytes obtained at plating consistent with a heterogeneous cell culture (50). It is, therefore, perhaps best to describe bulk cultures as consisting of neural precursor cells (NPCs), which are a mix of stem cells and progenitors. Recent studies have show that in the adult brain the stem cell may in fact be the ependymal cell lining of the ventricle (36). These cells divide very slowy and often asymmetrically *in vivo* but give rise to more rapidly dividing progenitors or transit amplifying cells. It is likely that the majority of stem cells within neurospheres are these transit amplifying cells, and the stem cell, by nature of slow and often asymmetric divisions, is relatively rare.

Where clonal studies show a restriction in phenotype, there is the likelihood that a progenitor has been isolated. The best studied of these is the oliogdendrocyte and type II astrocyte progenitor (O2-A) which is restricted to producing these two types of cells at the expense of neurons and has been extensively characterised (57, 85). However, it is always possible that under different culture conditions or following transplantation, progenitor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cells can express a wider range of phenotypes than seen spontaneously *in vitro* ((24) and Brüstle in this issue). Moreover, recent studies have suggested that neural stem cells derived from the CNS may also be able to give rise to cells which normally derive from the neural crest, including Schwann cells and smooth muscle (47). Experiments which have shown that quiescent nuclei from fully differentiated cells can, when transferred to an oocyte, re-express embryonic genes and generate a new organism or clone (90) suggest that our current ideas of cells with limited phenotypes may have to be radically modified in the future. Although an extreme view, it could be argued that many cell types, but in particular precursor cells capable of division, may undergo "de-differentiation" to a more primitive state given the right circumstances. The recent findings that cloned mouse neural stem cells may be able to re-populate bone marrow and transform into blood cells lends further credence to this suggestion (5). Another factor to consider when interpreting data showing restricted phenotypes arising from a given cell, is that selective cell death may eliminate a specific class of cells generated from a clone, giving the impression of uni-potency where multi-potency in fact existed (87). In summary, whether generated from a single cell or population, the phenotypic potential of precursor cells grown *en mass* will depend upon a complex interaction between both their origins, and the environment in which they are placed.

Human neural stem cells

While a large amount of evidence is accumulating to suggest that neural stem cells exist in both the developing and adult rodent CNS, what about human tissues? A cluster of recent papers show that cells derived from the embryonic human CNS can be isolated and cloned in culture ((21) and see Snyder *et al* this symposium), grown for extended periods of time *in vitro* (13, 82), and incorporate into the developing rodent CNS ((8) and see Brüstle *et al*, this symposium).

However, the first evidence that FGF-2 could induce the proliferation of human CNS precursors came over 3 years ago from the work of Buc-Caron (9). Various brain regions from embryo's between 5 and 12 weeks of age were used. Cells were expanded in the presence of FGF-2 for short periods of time as a monolayer and on a Matrigel substrate (which contains both laminin and trace amounts of growth factors) in the absence or presence of serum. Proliferation was recorded for up to six weeks although there was no quantification of population doublings. A high percentage of cells were found to express nestin, a marker of early neuroepithelial precursors (40). In addition, markers for neurons, astrocytes and oligodendrocytes were also found suggesting that a neural stem cell might be dividing in these cultures, but not ruling out the possibility that a multitude of different non multi-potent lineages were dividing along side each other. We found that cells isolated from the mesencephalon of older human embryo's (>13 weeks), but not younger ones, could be induced to divide with EGF and grew as neurospheres which appeared similar, in appearance at least, to those seen in the rodent (77). Due to problems with inducing precursors to divide from early human embryos with EGF, we began to use FGF-2 and showed that it was possible to stimulate the division of early human precursors as neurospheres with this mitogen, that it acted synergistically with EGF, and that expanded populations of cells could again give rise to neurons, astrocytes and oligodendrocytes at early passages (76). Another report has described similar problems with regard to inducing the division of human neural precursors from young cortical tissues (6-8 weeks) with EGF alone, and needed to use both 5% horse serum in combination with insulin like growth factor-I to expand the cells as neuropsheres (14).

Oligodendrocytes are often seen to differentiate from FGF-2 responsive human precursors, but have been most fully characterised by Murray *et al* (48). They showed that FGF-2 responsive neural precursors could be isolated from 8 week old human fetal CNS tissue and formed spheres in culture when seeded onto fibronectin coated flasks. Cells required brief exposure to serum to attach, but then remained in defined serum free condi-

Figure 3. (Opposing page) Serially applied FGF-2 and BDNF allow expansion and survival of neurons arising from the human subependymal zone (SZ). (**A**) Cell outgrowth from adult human SZ at 9 weeks in vitro. The explant was exposed to FGF-2 for a week in the presence of [^{3}H] thymidine, then to BDNF for 2 months, and subsequently fixed and stained for the neuronal marker microtuble associated protein-2. (**B**) Two of these MAP-2+ cells incorporated [^{3}H] thymidine during their first week in vitro (aggregations of silver grains denoted by arrows), indicating mitotic neurogenesis during the period of FGF-2 exposure. (**C**) High power of cell in A and B (asterisk), stained for MAP-2. (**D**) After [^{3}H] thymidine autoradiography. (**E**) Another outgrowth from an adult temporal SZ explant, 7 weeks in culture. This sample was also raised in FGF-2 followed by BDNF, and then subjected to confocal imaging of the fluorescent signal emitted by the calcium indicator dye fluo-3; this was perfomed to assess neuronal responses to depolarising stimuli. (**F**) Baseline fluo-3 fluorescence signal from the neurons indicated in phase in E (arrows). (**G**) Immediately after depolarization by 60mM KCL. A similar response to 10μM glutamate was observed (not shown). Bar in A-D = 25μm; bar in E-G = 75μm. From (52) with permission.

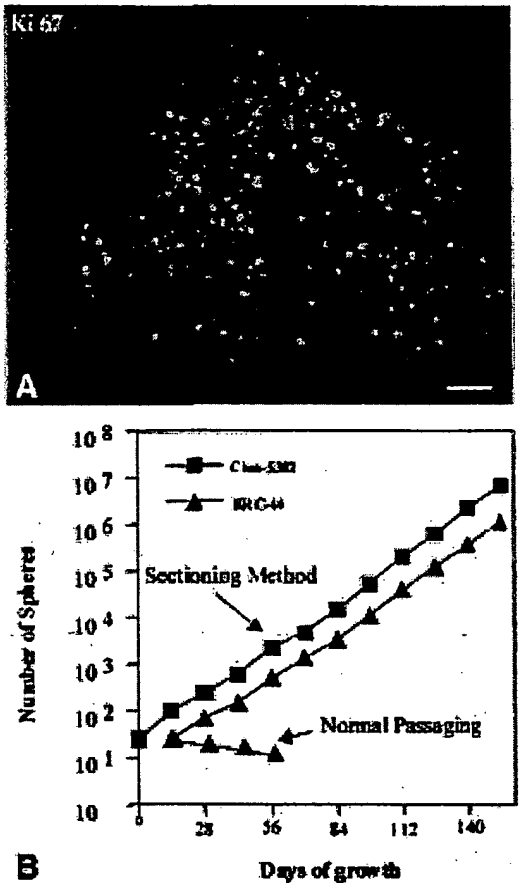

Figure 4. (A) Photomicrograph of a whole human neurosphere grown in FGF-2 for 100 days and stained for Ki67 which labels the nuclei of dividing cells (green). Note the many positive cells, particularly around the periphery of the sphere, suggesting extensive proliferation is occurring even at this late stage in culture. (B) Systematic counts of the total number of spheres at each passage (grown in FGF-2 and serum free media) was established using conventional methods where the spheres are triturated with a narrow pipette tip (normal passaging) or sectioned into quarters with a scalpel (sectioning method of passaging). Expansion of total cell number is only seen with the latter method under these conditions. Cells were derived from the cortex of an 7 week (BRC-44) or 22 week (Clon-5382) human embryo (16).

tions where active proliferation took place. Removal of FGF-2 allowed the differentiation of spheres which formed "chains" of migrating cells which were PSA-NCAM positive. The majority of these cells developed into neurons, but a small number of oligodendrocytes were also seen which could be increased in number by the addition of thyroid hormone T3.

Together, these papers show "proof of concept" that human NPCs (HNPCs) can be maintained in culture and may be similar to their rodent counterparts, but issues of regional specificity, long term growth and clonal analysis were not addressed in these papers. Interestingly, small numbers of dividing cells have also been found in cultured hippocampal slices taken from the adult human brain (39). In recent extensions to these original observations, brain derived neurotrophic factor (BDNF) has been shown to induce outgrowth from newly generated, adult derived human neurons responsive to FGF-2 [(52); Figure 3]. These studies, taken together with recent reports showing that dividing cells exist in the adult human hippocampus *in vivo* (19), raise the exciting prospect of generating neural precursor cell populations from the fully mature human CNS (27).

The Holy Grail of limitless neural tissue?

Aside from the obvious interest in HNPCs as a window on neural differentiation, they are also sought after by pharmaceutical companies and neural transplantation programmes, providing they can be significantly expanded and induced to mature into functioning neurons. A common (and incorrect) assumption, is that once a precursor is isolated and induced to divide in culture, it will continue to grow indefinitely. However, those who regularly work with dividing cells in culture know that this is not the case. Somatic, non-transformed cells are mortal. Following a set number of population doublings, ranging between 30 and 50 for most tissues, they enter replicative senescence and stop dividing (for review (32)) which may be a direct result of protective telomeres being eroded from the ends of chromosomes at each division (6). This has been well characterised in many cell types, but has not been widely discussed in the context of NPCs. There are few reports where the actual numbers of NPCs harvested at each passage have been analysed over long periods of time, from either rodent or human tissues. This is required in order to establish approximately how many doublings have occurred within a population. In one study, log growth of mouse EGF responsive precursor cells grown as neurospheres was recorded (62). We attempted to reproduce these results under identical conditions using rat in addition to mouse tissues. Although the mouse neurospheres could be grown for long periods of time, cells within the rat neurospheres consistently underwent what appeared to be replicative senescence at approximately 8 population doublings (80). This is remarkably similar to the

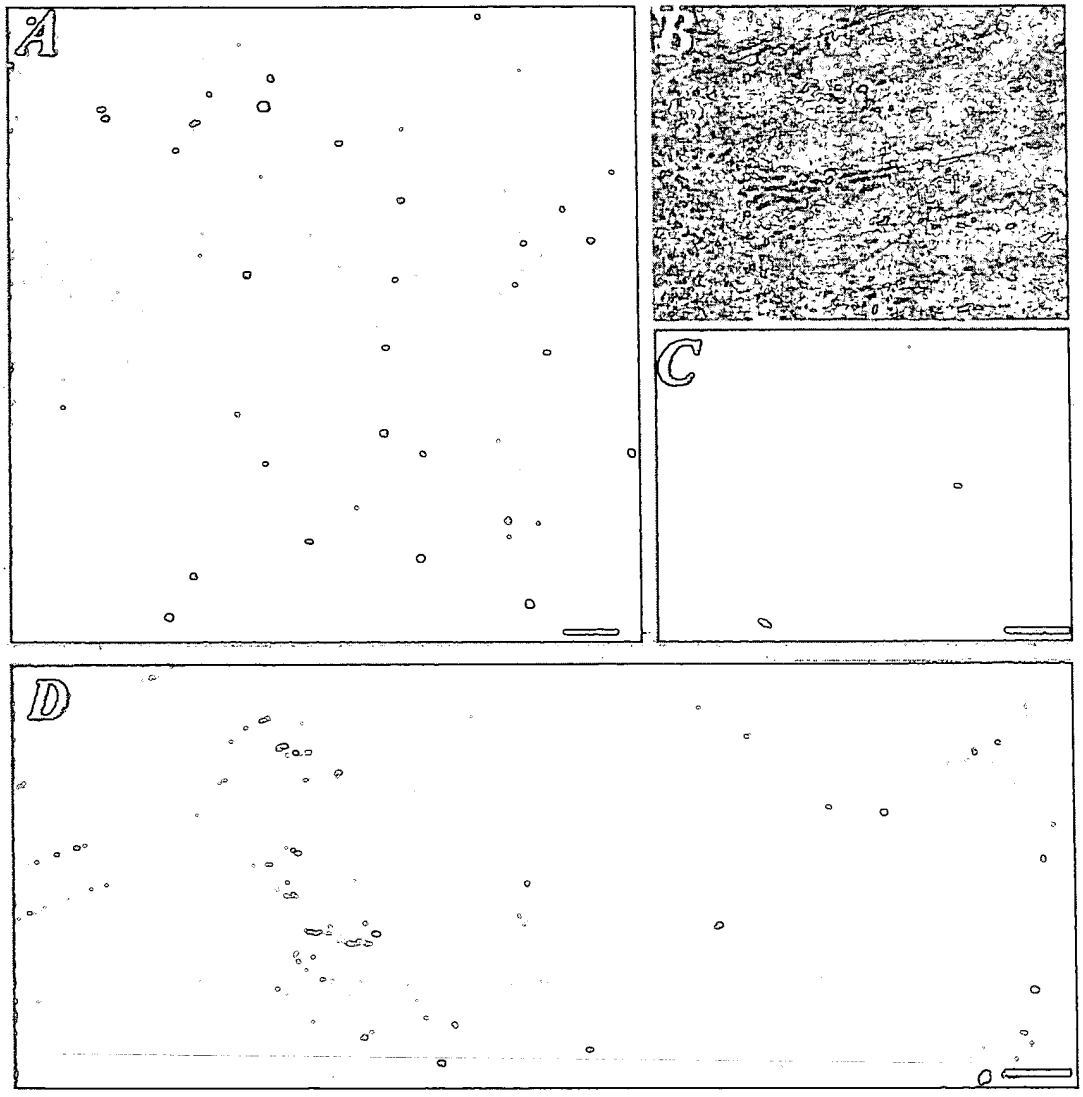

Figure 5. Whole spheres (grown for 150 days in FGF-2) were plated onto lamanin coated coverslips in the absence of FGF-2 and left for 14 days. (A) Radial processes could be seen to have extended from the core of some spheres along which extensive migration of neuronal precursors could be seen. Stained for TuJ1 (red) , a specific neuronal marker (45). Scale bar = 70μm. (B) and (C) show the same field under phase or stained for TuJ1 respectively from a similar culture. Note the small neuronal cells along the radial processes. Scale bar = 25μm (D) Lower power image showing the region of a similar human neurosphere (plated to the left and out of view) and demonstrating the large number of neurons and astocytes generated by a single sphere. Glial fibrilary acidic protein (GFAP), an astrocyte marker, in green and TuJ1 in red. Scale bar = 100μm.

number of divisions which O-2A progenitor cells are able to undergo before spontaneously differentiating in culture (56), and may be due to an intrinsic timing mechanism which limits their number of divisions. Interestingly, similar studies using rat FGF-2 responsive precursors grown attached to a substrate appear in some cases to grow for much longer periods (49, 59) although cumulative cell counts are not given for these cultures

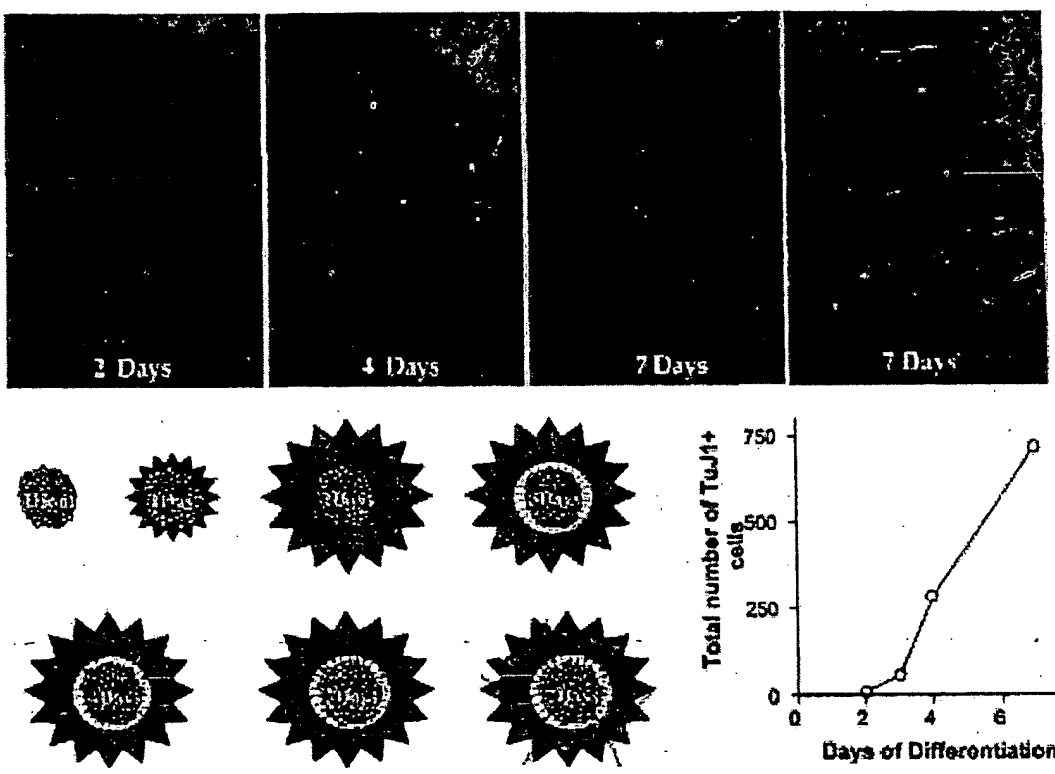

Figure 6. Photomicrographs show the sequential appearance of astrocytes (green) followed by neurons (red) when whole human neurospheres are plated and allowed to differentiate as described in Figure 5. The first cells to emerge from the sphere are almost exclusively astrocytes but are followed after a latency of 24 to 48 hours by migrating neurons. The last panel shows staining of the same sphere for neurofilament 70, another specific neuronal marker. The schematic shows this process in a stylised form and the graph shows cell neuronal cell counts from around the sphere at increasing stages of differentiation.

beyond 6 weeks of growth. In our hands, human neurospheres only underwent approximately 4 to 6 population doublings using standard passaging techniques (76). Murray et al (48) indicate that cultures of human neurospheres could be expanded for up to 7 months through 8 passages but provide no information on either the growth rates during this time, or the differentiation potential of the cells following extended expansion.

As the amounts of growth that we, and others, were achieving in culture appeared to be well below the Hayflick limit of 50 population doublings (32), we set out to investigate factors which might be responsible for the lack of division at later time points. After attempting a number of modifications to the culture medium we did discover that heparin sulphate was crucial for the actions of FGF-2 on free floating neurospheres (but interestingly not so important for the growth of plated precursor cells) (10). However, this, or other modifications, had no effect on the senescence seen at later stages of growth. We next turned to the way in which the cells were passaged. As the spheres become larger they begin to lose the ability to pass nutrients to their cores and as such begin to slow their growth rates. At this stage the conventional method for keeping the culture going is to break the sphere down to single cells and then re-plate into new flasks. If enzymes such as trypsin are used during this process, the continuation of growth is often very slow following passaging, perhaps due to the removal of vital receptors. Furthermore, many cells appear to spontaneously differentiate following trypsinisation, further slowing the rate of growth. If no enzymes are used new spheres will form slowly over time but there is generally over 50% cell death and so half the culture is lost, significantly reducing its overall expansion rate.

There are some reports where cells in clusters of freshly plated rodent CNS precursors remain mitotically active, whereas isolated cells within the same cultures do not often divide (26). This, and the fact that membrane associated factors can stimulate division of NPC's (84) suggested that cell/cell contacts and the extracellular matrix are vital for efficient cell division *in vitro*. In light of these studies, and our previous observations that cell density is crucial to efficient proliferation of NPC's (78), we developed a novel passaging method in which whole human neurospheres, grown from 8 or 20 week old cortex in a combination of EGF, FGF-2 and heparin, were sectioned into quarters rather than mechanically dissociated. Cells within each quarter maintained contact with each other following passaging, and each quarter rapidly rounded and began to grow, generally reaching the size of the mother sphere within 14 days (82). Using this simple method, long term exponential expansion could be achieved for up to 200 days and spheres contained large numbers of dividing cells (Figure 4). The overall growth rate was slow compared to rodent cells with a population doubling period of approximately 4-5 days. At later passages, cells divided in response to either EGF or FGF-2 alone, although in FGF-2 the neurospheres often formed disc structures which adhered to the culture dishes (82). All cultures stopped dividing between 250 and 300 days and appeared to either undergo senescence and remain undifferentiated, or spontaneously differentiated into either a neuron or an astrocyte. At this stage, most cultures had undergone approximately 30 population doublings (a theoretical increase of 1 billion fold) and it is possible that the natural Hayflick limit for these cells had been reached leading to cessation of division. This data is similar to work on propagating human astrocytes, which have been shown to achieve 40 population doublings before reaching replicative senescence (53). The fact the cells stopped dividing is important as it shows that the cells have not oncogenically transformed in culture.

The remarkable aspect of these human cells was that they continued to produce over 40% neurons when allowed do differentiate at either 50 or 150 days of expansion (Figures 5, 6). The majority of these were GABAergic, a topic discussed further in a later section This is in direct contrast to mouse neurospheres grown under very similar conditions which generate less than 5% neurons even at early passages (2), and rat neurospheres in which the percentage of neurons decreases with each passage to less than 1%, up until senescence at around 5 weeks (Rosser and Svendsen, in preparation). Furthermore, whereas human FGF-2 responsive

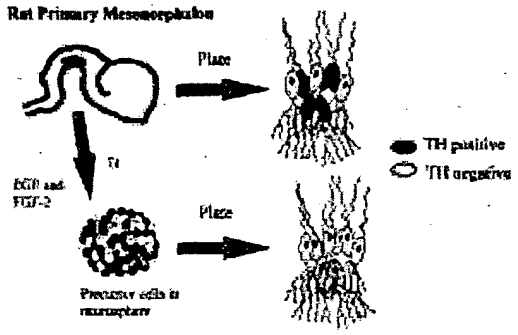

Figure 7. Rat primary mesencephalon contains many dopaminergic neurons, which express tyrosine hydroxylase when plated and cultured for 7 days (TH; red cells). However, if the same cells are expanded for 7 days and then plated, the number of TH positive neurons reduces dramatically, suggesting that the dividing cells lose their capacity to generate TH neurons spontaneously.

neurospheres grown for short periods of time gave rise to some oligodendrocytes, very few were found to differentiate from long term human neurospheres (82). This was again in contrast to mouse and rat studies where large numbers of oligodendrocytes can be found at all passage times and are able to re-myelinate lesions of the spinal cord (30). Efforts to generate more oligodendrocytes from human precursor cells are currently underway. Interestingly, canine and rodent "oligospheres" have been described recently using a method where the culture is supplemented with B104 conditioned medium (3, 92, 93). Although it is not clear whether this medium will also be able to support human neurospheres with the capacity to differentiate into large numbers of oligodendrocytes, there is some evidence that conditioned medium from other cell lines may support such cultures (Zhang and Duncan, personal communication). It is of interest that following immortalization with v-*myc*, human neural precursors have been cloned and shown to generate either neurons alone, or neurons and astrocytes but not oligodendrocytes (66), which would appear to be very similar to the long term human neursophere cultures described here.

Our attempts at clonal analysis using human NPC's have so far been unsuccessful, and we have therefore been unable to determine the potential of individual cells. This possibly reflects an extreme sensitivity of single HNPCs in a culture dish, which may require specific additions to the medium in order to survive and divide. Until we perform these analyses it is possible that we are growing either (i) a bi-potent precursor capa-

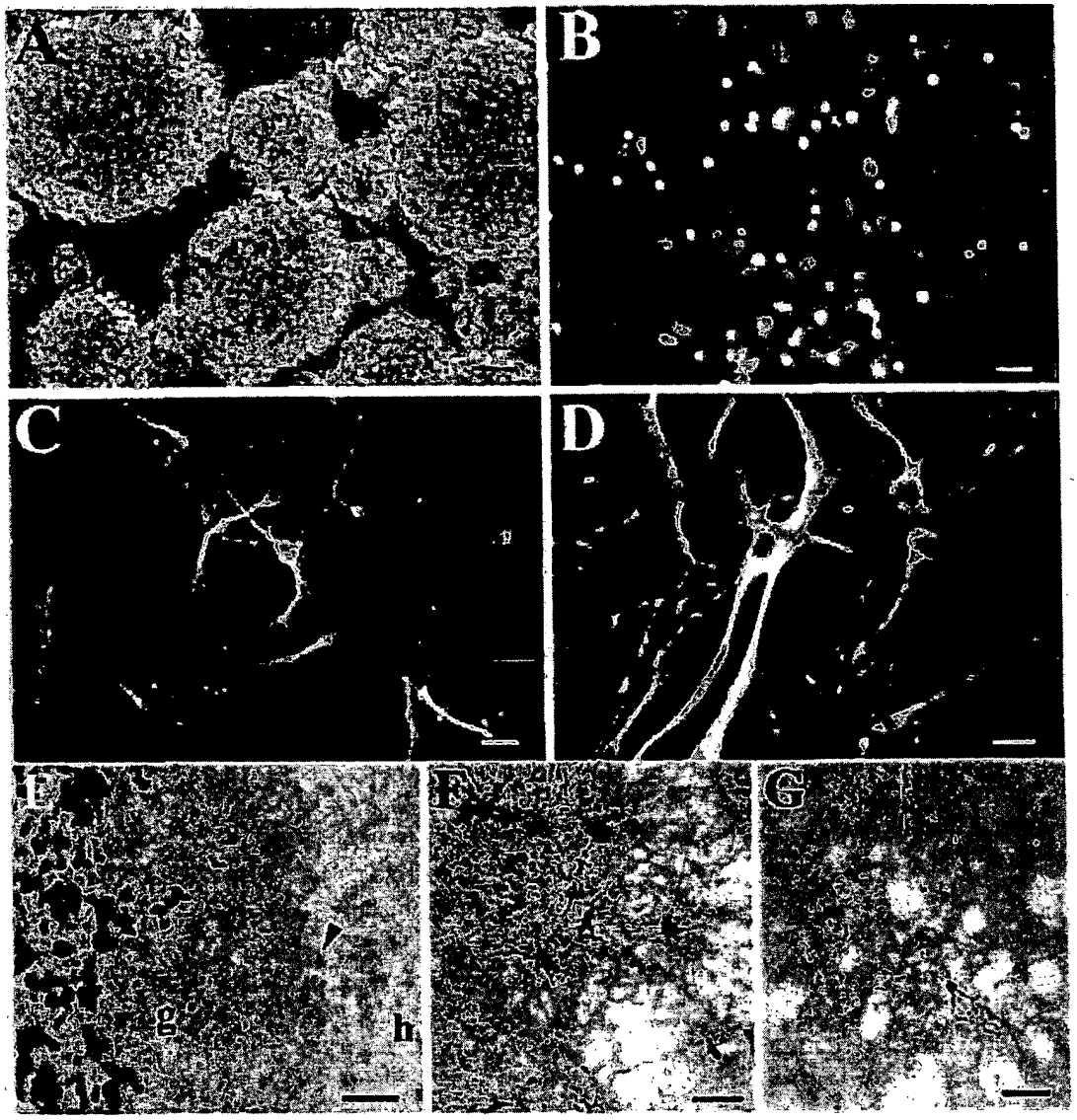

Figure 8. Human NPCs isolated from a 22 week old cortex were expanded *in vitro* for 21 days as neurospheres (**A**) with EGF and FGF-2. (**B**) Following pulsing with BrdU, dividing cells were plated and stained at 7 days with antibodies against BrdU (**B**) TuJ1 (**C**) or GFAP (**D**). Note the large number of BrdU positive cells and mixture of neurons and astrocytes labelled. (**E**) BrdU labelled cells injected into the 6-OHDA lesioned striatum began to migrate out from the core of the graft (g) at two weeks post transplantation. As they enter the host tissue (h) they have a weaker stain (arrowhead) suggesting that they are actively proliferating while migrating. (**F**) Over the next 20 weeks many cells migrated from the graft core and matured into astrocytes labelled here with human GFAP, a specific marker for human astrocytes. This human GFAP antibody will label highly reactive rodent astrocytes following a lesion, but not 20 weeks after the lesion. All sham grafted animals were negative for human GFAP staining at 20 weeks. (**G**) Staining for TH which labels dopaminergic neurons revealed a number of positive cells in two animals which had extensive ramifications into the striatum. These cells were found around the graft site in close proximity to human Tau stained neurons (not shown) and were not seen in any of the sham grafted animals. Taken from (76) with permission.

ble of making neurons and astroctyes, (ii) two unipotent cells, one able to produce neurons and the other astrocytes or (iii) a multpotent precursor which would produce oligodendrocytes under different culture conditions.

We were also interested in establishing whether rat neurospheres could be expanded beyond 5 weeks using this new sectioning method. However, in all cases the rat neurospheres underwent senescence irrespective of the passaging technique. Furthermore, they often appeared to adhere more readily to the surface of the culture dishes, and undergo spontaneous differentiation at later passages (Svendsen *et al*, unpublished observations). These studies highlight the differences between human and rodent neural precursor cell growth in culture and emphasise the need for detailed characterisation of culture systems for each species.

Very recently, another group have shown a similar problem maintaining dividing HNPCs for long periods of time under normal culture conditions, but have shown that leukaemia inhibitory factor (LIF) added to the medium will allow continual cell growth to occur, albeit at a slower rate than in our system (13). LIF acts through the gp130 signal transducing subunit and is also required for the continual growth of embryonic stem cells, and appears to maintain these cultures in a proliferative state by preventing differentiation (73). Although best characterised as a growth factor of the immune system, it is also now seen as having a wide range of effects on the CNS (51) most of which have focussed on its ability to promote differentiation rather than prevent it (64). Whether the inclusion of LIF in the medium and our method of sectioning share a common mechanism with regard to extended growth of human NPC's, and whether the type of cell propagated in both cases is the same, is presently unclear. Interestingly, using LIF in the medium results in the continual generation of small numbers of oligodendrocytes, in contrast to the sectioning method where none can be found. Continual modifications to culture mediums and passaging techniques will, no doubt, result in many other methods of long term growth for HNPCs over the coming years.

Induction of neural phenotypes.

Establishing that neural precursors can be expanded for long periods of time *in vitro* is exciting. However, what phenotype do neurons arising from such cells express? The consensus for rodent precursors, generated in many different ways from a variety of embryonic or adult brain regions, is that they appear to be extremely plastic at early passages, differentiating into neurons, astrocytes and oligodendrocytes in response to growth factors and other additions to the medium and express a variety of developmentally regulated genes (for thorough reviews see 11, 41). There is also general agreement from the majority of reports on this subject, that newly generated neurons derived from expanded populations of precursor cells are to a large degree GABAergic. However, following transplantation back into the adult rodent CNS these cells appear to respond to the local environment and produce a wider range of phenotypes, as discussed below.

Attempts to induce dopamine neurons from precursor cells have been the focus of many studies, due mainly to the interest in producing such cells as a source of tissue for transplantation therapy in PD (79). It has been shown that in the presence of FGF-2, precursor cells isolated from the developing rodent mesencephalon will undergo at least a few divisions while retaining the capacity to generate another dopamine neuron, but subsequent divisions lead to precursors which can no longer spontaneously generate dopamine neurons (7, 10) (Figure 7). This allows short, but not long term expansion of the primary dopamine neuroblasts. There appears to be evidence that Interleukin 1 is able to induce the dopamine phenotype in a large proportion of mesencephalic rodent precursors, and that this effect can be significantly enhanced by adding membrane fragments, conditioned medium, IL-11, LIF and growth factors such as GDNF (42). However, induction of the dopaminergic phenotype has been more difficult using the same method on HNPCs at later passages (13). We have assessed the effects of this cocktail and sonic hedgehog and FGF-8 on induction of dopaminergic phenotypes from the human precursors, previously shown to be involved in this process *in vivo* (91), with negative results (Svendsen *et al*, unpublished observations). Does this mean that the synchronous growth of HNPCs leads to a cell which can no longer respond to cues vital to differentiation? We think this unlikely due to recent studies where these cells have been transplanted into either the embryonic or adult rodent CNS as described in the next section. Furthermore, transcription factors such as *Nurr1* and *Ptx3*, have been discovered which may play an important role in the induction and phenotypic maturation of dopamine neurons (67, 71). Over-expression of these transcription factors in neural precursor cell populations, in combination with the appropriate growth factor treatments may yet lead to the controlled generation of dopamine neurons.

Transplantation of human NPC's into the developing or adult CNS

When primary rodent tissues, which contain a high proportion of precursor cells, are transplanted back into the developing CNS they appear to migrate widely with little preference for the location from which they originated (12, 20). In other studies hippocampal precursor cells have been expanded for significant lengths of time *in vitro* and subsequently injected back into various regions of the adult CNS. When placed in the rostral migratory stream they have been shown to migrate along with host cells and, upon arrival in the target zone, differentiate into neurons in a site-specific manner, even generating dopamine neurons in some cases (75). However, in other non-neurogenic brain regions less cells survive and differentiate (75). These, and many other rodent precursor transplantation studies, have been reviewed in detail recently (24) and are discussed further in this symposium (see Brustle chapter), but what about the transplantation of human neural precursors?

Transplantation of HNPCs into either the embryo or neonate are discussed elsewhere within this symposium (Brustle and Snyder chapters). Here transplantation into the adult CNS will be considered. In one of the first studies, cells isolated from the cortex of a 12 week old human fetus were exposed to FGF-2 for 11 days *in vitro*, infected with an adenovirus encoding the *lacZ* gene and then transplanted into the striatum of immunosupressed rats (65). Although no cell survival was found in grafts of 600,000 cells or less, three out of four animals with a million cells or more transplanted had surviving grafts at three weeks, which contained a number of beta galactosidase expressing neurons. As the original cultures had not been passaged, and the cells within the grafts were not labelled with mitotic markers to prove they had divided in culture, it is possible that these neurons may have been primary cells from the initial culture, or cells which had not undergone division. We addressed this issue directly by transplanting two populations of human precursors into the striatum of rats with 6-OHDA lesions of the dopaminergic neurons within the ventral mesencephalon. EGF responsive cells isolated from the ventral mesencephalon were grown for either 10 or 28 days and then transplanted. We found that whereas the 10 day old cultures gave rise to solid grafts which contained a number of dopaminergic neurons, cultures grown for 28 days had no discernible graft mass although there was the occasional dopaminergic neuron within the lesioned striatum (77). Thus, cells which had been passaged to remove primary neurons appeared very different to cells which still contained primary neurons, perhaps relating to the loss of appropriate differentiation signals with increasing divisions as discussed in the previous section. In a later study we showed that when expanded populations of HNPCs (between 14 and 28 days of growth, passaged every 7 days) isolated from the developing cortex were transplanted into lesioned animals, solid grafts were found at 2 weeks post transplantation, but these reduced in size over time such that by 20 weeks only a thin strip of cells remained (76). It should be noted that in this study between 200,00 and 500,000 cells were transplanted, and that higher cell densities may result in a large graft mass remaining at later survival times. Using a variety of human-specific antibodies, we were able to demonstrate that a large number of cells had migrated out and differentiated into astrocytes while a far smaller percentage had differentiated into neurons (Figure 8). Interestingly, in two animals a significant number of these neurons had become dopaminergic by 20 weeks and were able to reverse rotational deficits associated with the lesion (76).

This study provides the first evidence that HNPCs can engraft into the adult CNS, mature into dopamine neurons and restore function in a rodent model of PD. Why did this only occur in a small number of animals? This in part reflects biological variability in terms of haplotype and HLA status in a cohort of wild type rats. We are currently exploring this further by transplanting HNPCs into immunodeficient rats and assessing other types of immune suppression in addition to cyclosporin, which has normally been used in our studies. However, the fact that some of these cells can develop into mature dopaminergic neurons following transplantation into this rat model of PD lends impetus for future studies in this area, and provides proof of concept that these HNPCs are capable of maturing into functioning dopamine neurons *in vivo*.

Conclusions

This review covers the recent developments in human neural progenitor and stem research, from basic developmental biology through to possible therapeutic applications. Recent evidence suggests that a human neural stem cell does indeed exist (21), although arguments over the percentage of these cells in various model systems will no doubt persist for many years. In strict semantic terms, cells within such cultures should be referred to as neural precursors. More specifically however, the term neural stem cell is a useful description (and appears in the title of this article), as it is then possible to relate them to embryonic, blood and skin stem cells which have been well characterised as stem cells

and have a more clearly defined biology (29, 54, 89). It is a particularly exciting time for stem cell biology since recent studies have shown that human embryonic stem (ES) cells, capable of producing all tissues of the body, can be grown in culture from human fertilised eggs or the gonadal tissue of the developing fetus (69, 86). ES cells will be important for studying the molecular mechanisms of human development, and it may soon be possible to derive neural stem cells from them directly, thus greatly facilitating the possibilities for genetic engineering ((72, 81) and see Isacson et al, this symposium). The ability to grow large populations of human NPC's will provide a source of tissue for many types of applications, from developmental biology through to drug screening and cell therapy. In addition, the recent demonstration that rodent neural stem cells may be able to form blood expands the possible therapeutic horizons further still (4). However, the characterisation of human cells in culture and elucidation of those genetic and epigenetic factors which control their fate, are a prerequisite for their use in a clinical setting. This is particularly pertinent, as there are often significant differences between these human cells and their rodent counterparts. The next few years should begin to see these goals being met.

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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin E. Reubinoff, et al.	**Examiner:**	Deborah Crouch, PhD
Serial No.:	09/970,543	**Art Unit:**	1632
Filed:	4 October 2001	**Confirmation No:**	1839
For:	EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM		

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION OF CLIVE NIELS SVENDSEN UNDER 37 C.F.R. §1.132

This is the exhibit marked as "Exhibit E" referred to in the Declaration of **CLIVE NIELS SVENDSEN**

By: _____ Dated: 04/13/05.

Exhibit E

Adv Tech Stand Neurosurg. 2003;28:3-89.

Recent advances in stem cell neurobiology.

Ostenfeld T, Svendsen CN.

MRC Centre for Brain Repair, University of Cambridge, Cambridge, UK.

1. Neural stem cells can be cultured from the CNS of different mammalian species at many stages of development. They have an extensive capacity for self-renewal and will proliferate ex vivo in response to mitogenic growth factors or following genetic modification with immortalising oncogenes. Neural stem cells are multipotent since their differentiating progeny will give rise to the principal cellular phenotypes comprising the mature CNS: neurons, astrocytes and oligodendrocytes. 2. Neural stem cells can also be derived from more primitive embryonic stem (ES) cells cultured from the blastocyst. ES cells are considered to be pluripotent since they can give rise to the full cellular spectrum and will, therefore, contribute to all three of the embryonic germ layers: endoderm, mesoderm and ectoderm. However, pluripotent cells have also been derived from germ cells and teratocarcinomas (embryonal carcinomas) and their progeny may also give rise to the multiple cellular phenotypes contributing to the CNS. In a recent development, ES cells have also been isolated and grown from human blastocysts, thus raising the possibility of growing autologous stem cells when combined with nuclear transfer technology. 3. There is now an emerging recognition that the adult mammalian brain, including that of primates and humans, harbours stem cell populations suggesting the existence of a previously unrecognised neural plasticity to the mature CNS, and thereby raising the possibility of promoting endogenous neural reconstruction. 4. Such reports have fuelled expectations for the clinical exploitation of neural stem cells in cell replacement or recruitment strategies for the treatment of a variety of human neurological conditions including Parkinson's disease (PD), Huntington's disease, multiple sclerosis and ischaemic brain injury. Owing to their migratory capacity within the CNS, neural stem cells may also find potential clinical application as cellular vectors for widespread gene delivery and the expression of therapeutic proteins. In this regard, they may be eminently suitable for the correction of genetically-determined CNS disorders and in the management of certain tumors responsive to cytokines. Since large numbers of stem cells can be generated efficiently in culture, they may obviate some of the technical and ethical limitations associated with the use of fresh (primary) embryonic neural tissue in current transplantation strategies. 5. While considerable recent progress has been made in terms of developing new techniques allowing for the long-term culture of

human stem cells, the successful clinical application of these cells is presently limited by our understanding of both (i) the intrinsic and extrinsic regulators of stem cell proliferation and (ii) those factors controlling cell lineage determination and differentiation. Although such cells may also provide accessible model systems for studying neural development, progress in the field has been further limited by the lack of suitable markers needed for the identification and selection of cells within proliferating heterogeneous populations of precursor cells. There is a further need to distinguish between the committed fate (defined during normal development) and the potential specification (implying flexibility of fate through manipulation of its environment) of stem cells undergoing differentiation. 6. With these challenges lying ahead, it is the opinion of the authors that stem-cell therapy is likely to remain within the experimental arena for the foreseeable future. In this regard, few (if any) of the in vivo studies employing neural stem cell grafts have shown convincingly that behavioural recovery can be achieved in the various model paradigms. Moreover, issues relating to the quality control of cultured cells and their safety following transplantation have only begun to be addressed. 7. While on the one hand cell biotechnologists have been quick to realise the potential commercial value, human stem cell research and its clinical applications has been the subject of intense ethical and legislative considerations. The present chapter aims to review some recent aspects of stem cell research applicable to developmental neurobiology and the potential applications in clinical neuroscience.

PATENTS

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This is the exhibit marked as "Exhibit F" referred to in the Declaration of **CLIVE NIELS SVENDSEN**

By: _____ Dated: 04/13/05.

nature biotechnology

Specification of motoneurons from human embryonic stem cells

Xue-Jun Li[1,2,4], Zhong-Wei Du[1,2,4], Ewa D Zarnowska[3], Matthew Pankratz[4], Lauren O Hansen[4], Robert A Pearce[3] & Su-Chun Zhang[1,2,4]

An understanding of how mammalian stem cells produce specific neuronal subtypes remains elusive. Here we show that human embryonic stem cells generated early neuroectodermal cells, which organized into rosettes and expressed Pax6 but not Sox1, and then late neuroectodermal cells, which formed neural tube–like structures and expressed both Pax6 and Sox1. Only the early, but not the late, neuroectodermal cells were efficiently posteriorized by retinoic acid and, in the presence of sonic hedgehog, differentiated into spinal motoneurons. The *in vitro*–generated motoneurons expressed HB9, HoxC8, choline acetyltransferase and vesicular acetylcholine transporter, induced clustering of acetylcholine receptors in myotubes, and were electrophysiologically active. These findings indicate that retinoic acid action is required during neuroectoderm induction for motoneuron specification and suggest that stem cells have restricted capacity to generate region-specific projection neurons even at an early developmental stage.

The generation of functional neuronal subtypes in the vertebrate central nervous system involves several steps, including the induction of the neuroectoderm from the embryonic ectoderm, patterning of the neural plate into complex regional compartments along rostrocaudal and dorsoventral axes, and differentiation of the regionalized progenitors into postmitotic neurons[1,2]. These developmental processes are orchestrated by interactions among prospective precursors and morphogens, such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), retinoic acid, sonic hedgehog (SHH) and Wnts, secreted from surrounding tissues in unique spatial and temporal orders[1–4]. On the basis of these developmental principles, mouse embryonic stem (ES) cells, isolated from the inner cell mass of a blastocyst embryo[5,6], have been induced *in vitro* to adopt a neuroectodermal fate[7–9], with subsequent differentiation into large projection neurons, such as midbrain dopaminergic neurons and spinal motoneurons, in response to specific sets of morphogens[10–12].

Human (h)ES cells can be maintained *in vitro* for a prolonged period with a stable genetic background and thus may provide a source of specialized human cells for biotechnological and clinical applications[13]. We differentiated hES cells (H1 and H9 lines) into neuroepithelial cells using our chemically defined adherent colony culture system[14]. Analyses of morphology and gene expression patterns revealed multiple stages in the differentiation to neuroectoderm. ES cells and those aggregated for 4 d did not express neuroectodermal transcription factors such as Pax6, Sox1 and Sox3. The first sign of neural differentiation was the appearance of columnar cells forming rosettes in the center of colonies 8–10 d after ES cells were removed from feeder cells for differentiation (**Fig. 1a,b**). The columnar cells in the rosettes, but not the flat cells in the outgrowth area, expressed Pax6 (**Fig. 1c**) but not Sox1 (**Fig. 1d**), which is the earliest neuroectodermal marker expressed during neural plate and tube formation[15]. With further culturing in the same medium for another 4–5 d, the columnar cells organized into neural tube–like rosettes with lumens (**Fig. 1e**) and expressed both Pax6 and Sox1 (**Fig. 1f–h**). Pax6 and Sox1 mRNA were expressed sequentially, as revealed by RT-PCR (**Supplementary Fig. 1** online). The same sequential expression of Pax6 and Sox1 was observed in the absence of FGF2 (**Supplementary Fig. 1** online). Cells at both stages expressed nestin[14], a common neural progenitor marker. Thus, differentiation of neuroectodermal cells from hES cells involves at least two easily identifiable stages, the $Pax6^+/Sox1^-$ columnar cells in the early rosettes 8–10 d after neural induction, and the $Pax6^+/Sox1^+$ cells forming neural tube–like late rosettes 14 d after induction.

Immunocytochemical analyses revealed that the rosette cells, which expressed Pax6 (**Fig. 1i**), Sox1 and nestin, were positive for Otx2 (**Fig. 1j,l**), a homeodomain protein expressed by fore- and midbrain cells, but negative for HoxC8 (**Fig. 1l**), a homeodomain protein produced by cells in the spinal cord. They were also negative for engrailed 1 (En1), which is expressed by midbrain cells (**Fig. 1k**). These results suggest that the neuroectodermal cells differentiated in the presence or absence of FGF2 possess a rostral character, similar to that initially acquired by neuroectodermal cells during early *in vivo* development[16].

Examination of dorsoventral markers indicated that the Pax6-expressing neuroepithelial cells did not express Olig2 and Nkx2.2, homeodomain proteins expressed in ventral neural progenitors[17,18]. Pax7, a dorsally expressed transcription factor, was uniformly expressed by cells surrounding and at the edge of the neural rosettes. At the late rosette stage, Pax7 was also expressed by some cells in the rosettes (**Supplementary Fig. 2** online). This intrinsic or default

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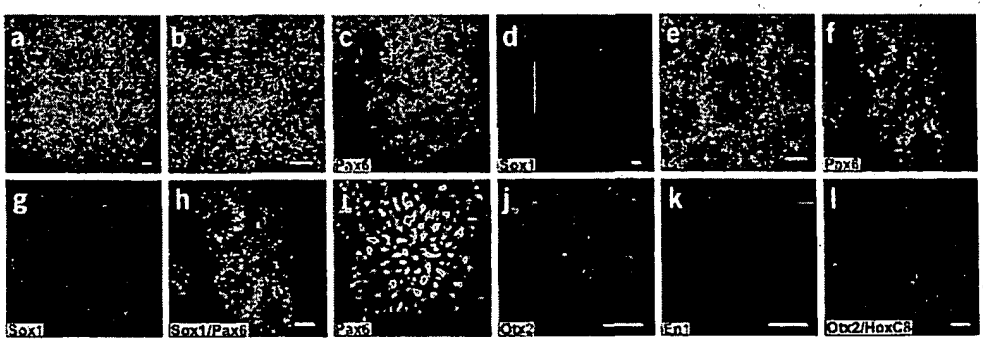

Figure 1 hES cell–derived neuroectodermal cells display rostral phenotypes. (a,b) ES cells, differentiated in FGF2 for 10 d, displayed small, columnar morphology in the colony center, and organized into rosette formations. (c,d) The columnar cells in the rosettes, but not the surrounding flat cells were positive for Pax6 (c) and negative for Sox1 (d). (e–h) By 14 d, the columnar cells formed neural tube–like rosettes (e) and were positive for both Pax6 and Sox1 (f–h). (i–k) The Pax6$^+$ cells (i) in the rosettes were also Otx2$^+$ (j) but were En1$^-$ (k). (l) Cells in the neural tube–like rosettes were positive for Otx2 and negative for HoxC8. Blue indicates Hoechst-stained nuclei. Bar, 50 μm.

dorsoventral patterning, together with the rostral identity of the neuroectoderm, indicates a need for caudalization and ventralization to generate spinal motoneurons.

We isolated hES cell–generated, Sox1$^+$ neuroectodermal cells in the rosettes through enzymatic treatment[14] and differentiated them on a laminin substrate in the presence of retinoic acid (0.001–1 μM) and SHH (50–500 ng/ml). Cells migrated out from the rosette cluster and extended neurites as early as 24–48 h after plating. By 14 d after plating, a large number of cells in the outgrowth area formed networks through their processes (Fig. 2a). Immunostaining analyses indicated that the majority of differentiated cells were positive for βIII-tubulin and MAP2. A large proportion (>50%) of them were also positive for Islet1 (Fig. 2a) and Lim3 (data not shown), transcription factors that are associated with motoneuron development[1,19]. However, very few cells in cultures plated from 1–3 weeks expressed HB9, a motoneuron-specific transcription factor[20] (Fig. 2a). A similar result was obtained when the neuroectodermal cells generated in the absence of FGF2 were used or when we used the suspension differentiation culture, as described for mouse ES cell differentiation[12].

The above observation suggests that the Sox1$^+$ neuroectodermal cells may be refractory for motoneuron induction. The cells expressing Sox1 may correspond to neuroectodermal cells in the neural plate or neural tube that are regionally specified[21]. This consideration led us to hypothesize that retinoic acid may promote caudalization and/or motoneuron specification before neuroectodermal cells express Sox1. We thus treated the neuroectodermal cells with retinoic acid (0.001–1 μM) at an earlier stage, that is, when columnar cells began to organize into rosettes and expressed Pax6. Cells treated in this way for 6–7 d displayed neural tube–like rosettes and expressed Sox1, indistinguishable from FGF2-treated cultures. We did not observe cells with

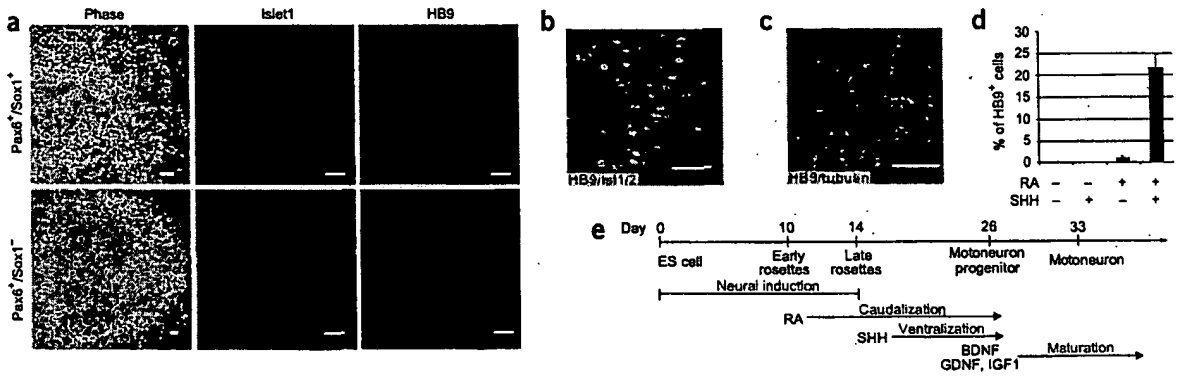

Figure 2 Generation of motoneurons from neuroectodermal cells. (a) Differentiation of Sox1$^+$ neuroectodermal cells for 2 weeks (1st row) revealed extensive neuronal generation in the outgrowth area, expression of Islet1, but few HB9$^+$ cells. Treatment of Pax6$^+$/Sox1$^-$ neuroectodermal cells (2nd row) resulted in extensive neurite outgrowth with few migrating cells, expression of Islet1 and a large proportion of HB9$^+$ cells. (b) About 50% of Islet1/2$^+$ cells differentiated from early neuroectodermal cells were also HB9$^+$. (c) HB9$^+$ cells were also positive for βIII-tubulin. (d) About 21% of the cells in the cluster were HB9$^+$ when the cultures were differentiated in the presence of both RA and SHH, whereas few HB9$^+$ cells were observed when cultured in either RA alone, SHH alone or neither. (e) Schematic procedures for motoneuron differentiation. hESCs were differentiated to early neuroectodermal cells in the form of early rosettes in 10 d. They were then treated with RA for 1 week and the neural tube–like rosettes were isolated through 3–5 d of differential adhesion and then adhered to the laminin substrate (around day 20) in the presence of RA and SHH for neuronal differentiation. Olig2$^+$ motoneuron progenitors appeared after 3 weeks, and HB9$^+$ motoneurons around 4–5 weeks after differentiation from ES cells. Trophic factors were added in the subsequent maturation process of motoneuron cultures. Blue indicates Hoechst stained nuclei. Bar, 50 μm.

neuronal morphology and expression of neuronal markers such as βIII-tubulin. After the rosette clusters were isolated through 3–5 d of differential adhesion and then adhered to the laminin substrate (2–4 clusters/coverslip), numerous neurites extended from the cluster as early as 24–48 h after plating, with fewer cells migrating out of the rosette cluster. By 14 d after plating, the neurite outgrowth area covered almost the entire (11-mm diameter) coverslip, although there were limited numbers of neuronal cell bodies in the outgrowth area (Fig. 2a). Immunocytochemical analyses indicated that the differentiated cells were positive for βIII-tubulin and MAP2. The majority of neurons were positive for Islet1/2 (Fig. 2a,b). Among the Islet1/2-expressing cells, about 50% were also HB9$^+$ (Fig. 2b), suggesting that these double-positive cells are motoneurons. The Islet1/2$^+$ and HB9$^-$ cells were likely interneurons.

HB9-expressing cells were first observed 6 d after the neuroepithelial cells had adhered to the laminin substrate for differentiation or about 4 weeks after hES cells were differentiated (Fig. 2e). The highest population of HB9$^+$ cells was observed a week later. The HB9$^+$ cells were largely localized to the cluster area, representing about 21% of the total cells in the cluster (Fig. 2a,d). A few cells in the outgrowth area, often close to the cluster, expressed HB9. In the absence of retinoic acid, or SHH, or both, there were few HB9$^+$ cells in the cluster (Fig. 2d). Almost all the HB9-expressing cells were stained with βIII-tubulin, a neuronal marker (Fig. 2c); cells in all the control groups (without retinoic acid and SHH, or with retinoic acid or SHH alone) were also positive for βIII-tubulin. Thus, treatment with retinoic acid at an early stage of neuroectodermal development is required for an efficient induction of spinal motoneurons.

To understand why retinoic acid induces the early but not the late neuroectodermal cells to differentiate into motoneurons, we first examined the effect of retinoic acid on caudalization of neuroectodermal cells. Treatment of early rosette cells (Pax6$^+$/Sox1$^-$) with retinoic acid (0.001–1 μM) or FGF2 (1–100 ng/ml) for 7 d resulted in the maturation of rosettes, that is, formation of multilayered rosette cells and expression of Sox1. RT-PCR analyses indicated that retinoic acid treatment resulted in decreased expression of *OTX2* and increased expression of HOX genes such as *HOXB1*, *HOXB6*, *HOXC5* and *HOXC8* in a dose-dependent manner (Fig. 3a). Genes expressed by more caudal cells were induced by higher doses of retinoic acid. FGF2 induced a similar set of caudal genes (Fig. 3a) although another caudal gene, *HOXC10*, was also induced by FGF2 but not by retinoic acid. However, treatment with FGF2 did not eliminate OTX2 expression (Fig. 3a), suggesting that FGF2 elicits a wide range of rostrocaudal gene expression. Treatment of late rosette cells (Pax6$^+$/Sox1$^+$) with retinoic acid for 1 week did not alter the HOX gene expression pattern induced by FGF2.

Immunostaining revealed that neuroectodermal cells that were treated with FGF2 or retinoic acid for 1 week from the early rosette stage were still Otx2 positive and HoxC8 negative. After treatment with retinoic acid for 1 week from the early rosette stage followed by culture in the neuronal differentiation medium, neuroectodermal cells stopped expressing Otx2 (Fig. 3c). They began to express HoxC8 protein after 6 d, with the majority of cells expressing it at 10–12 d after differentiation (Fig. 3d). Almost all the HoxC8$^+$ cells were βIII-tubulin$^+$ neurons (Fig. 3e). In contrast, late rosette cells treated with retinoic acid for 1 week and then allowed to differentiate for 2 weeks, yielded few HoxC8$^+$ cells, although Otx2-expressing cells were decreased (data not shown). Thus, treatment of early but not late neuroectodermal cells with retinoic acid results in efficient caudalization with expression of HoxC proteins, which are associated with spinal motoneurons[22].

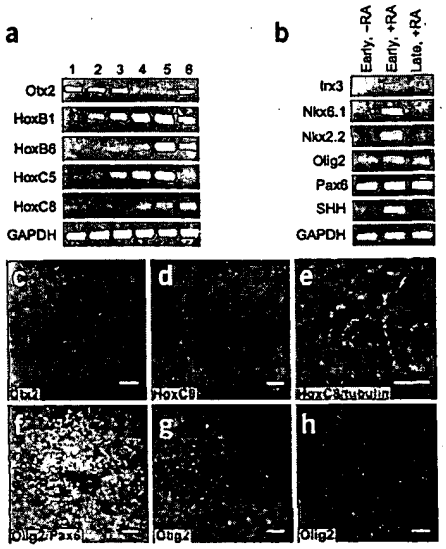

Figure 3 Effect of RA, FGF2 and SHH on neuroectodermal cells. (a) RT-PCR analyses indicated changes of rostrocaudal genes from early rosette cells that were cultured with retinoic acid (μM) or 20 ng/ml of FGF2 for 1 week in the neural induction medium. Lanes 1–5, retinoic acid (μM), 0, 0.001, 0.01, 0.1, 1.0, respectively; lane 6, FGF2. (b) Comparison of homeobox gene expression in early and late neuroectodermal cells treated with RA 0.1 μM for 1 week. (c,d) The early neuroectodermal cells, treated with retinoic acid and then allowed to differentiate for 12 d, became mostly negative for Otx2 (c) but positive for HoxC8 (d). (e) All the HoxC8$^+$ cells were βIII-tubulin$^+$. (f) The Pax6-expressing neuroectodermal cells were negative for Olig2. (g) After treatment with retinoic acid for 1 week and differentiation for 2 weeks in the presence of SHH (100 ng/ml), many cells expressed Olig2. (h) Few Olig2$^+$ cells were observed when late neuroectodermal cells were treated with retinoic acid and then allowed to differentiate under the same culture conditions. Blue, Hoechst stained nuclei. Scale bar, 50 μm.

Motoneurons are specified from progenitors in the ventral neural tube, and SHH is a key molecule for inducing ventral neural cell types[1,2]. The hES cell–derived neuroectodermal cells, whether they were Pax6$^+$ or Sox1$^+$, did not express Olig2 (Fig. 3f) or Nkx2.2 (data not shown). When the Pax6$^+$/Sox1$^-$ neuroectodermal cells were cultured in the presence of retinoic acid for 1 week, then isolated and allowed to differentiate for another 2 weeks in the absence of SHH, very few cells expressed Olig2. However, in the presence of SHH (50–500 ng/ml), many cells expressed Olig2 in the nuclei (Fig. 3g). In contrast, Pax6$^+$/Sox1$^+$ neuroectodermal cells, differentiated for 2 weeks under the same conditions, generated few Olig2$^+$ cells (Fig. 3h). Thus, neuroectodermal cells, treated with retinoic acid at an early but not a late stage, can be efficiently induced to a ventral neural progenitor fate in response to SHH.

To further discern why early retinoic acid treatment is required for motoneuron specification (Fig. 3a), we examined the expression of class I (Irx3, Pax6) and class II (Olig2, Nkx2.2, Nkx6.1) homeodomain molecules that are important in refining progenitor domains in the ventral neural tube[1,2]. Retinoic acid induced a much more robust expression of SHH and class II molecules, particularly Olig2 and Nkx6.1, in the early than in the late neuroectodermal cells (Fig. 3b). This suggests that early neuroectodermal cells are more responsive to retinoic acid in upregulating the expression of SHH and class II factors, which are essential for motoneuron specification.

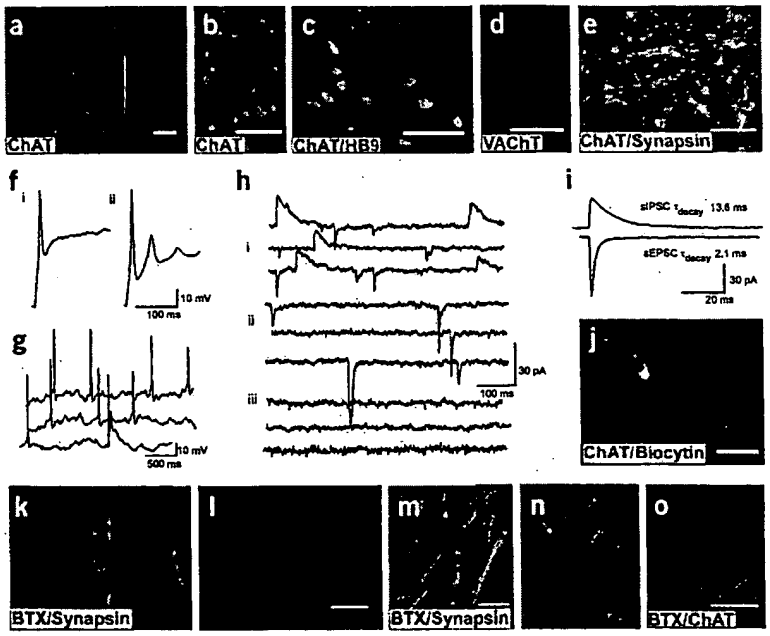

Figure 4 Maturation and functional properties of *in vitro*–generated motoneurons. (a,b) ChAT-expressing cells were localized mainly in the cluster (a), and were large multipolar cells (b). (c) Confocal analyses showed colocalization of ChAT in the soma and processes and HB9 in the nuclei in a 3-week culture. (d) Most cells in the cluster expressed VAChT. (e) Many ChAT$^+$ cells were also positive for synapsin in somas and processes after 5 weeks in culture. (f–j) Electrophysiological characterization of *in vitro*–generated motoneurons was evaluated by whole-cell, patch-clamp recording. Action potentials evoked by depolarizing current steps (0.15 nA) in neurons maintained for 42 DIV. Resting membrane potential (Vm) –59 mV (fi) and –70 mV (fii). (g) Spontaneous action potentials in a neuron maintained for 42 DIV, Vm –50 mV. (h) Spontaneous inward and outward synaptic currents at –40 mV using K-gluconate–based pipette solution under control conditions (hi). Bath application of bicuculline (20 μM) and strychnine (5 μM) blocked outward currents (inhibitory postsynaptic currents (IPSCs) hii). Subsequent application of AP-5 (40 μM) and CNQX (20 μM) blocked the remaining inward currents (excitatory postsynaptic currents (EPSCs) hiii). (i) Averaged sIPSCs and sEPSCs from the cell illustrated in panel h. (j) Double immunostaining for biocytin (from the recording electrode) and ChAT. (k–o) Further functional analysis was assessed using motoneuron-C2C12 myoblast cocultures. After 4 d of coculture, synapsin-expressing neurites made contact with myoblasts and myotubes, and patched α-BTX staining was observed on the myotubes (k,l). (m) After 2 weeks of coculture, synapsin positive neurites were very extensive and the α-BTX clusters were more obvious. A 0.3-μm confocal section derived from a Z-stack (arrows in m and n) showed colocalization of synapsin$^+$ axons and α-BTX stained acetylcholine receptors (n). (o) The myotubes with patched α-BTX staining were innervated by ChAT$^+$ fibers. Blue indicates Hoechst stained nuclei. Bar, 50 (a–e, and j) and 30 μm (k–o).

After treatment with retinoic acid at the early stage, neuroectodermal cells were enriched[14] and then allowed to differentiate into motoneurons by culture in the presence of SHH (100 ng/ml) for 6 d and then in neuronal differentiation medium with the addition of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF1) and a low concentration of SHH (50 ng/ml) (**Fig. 2e**). Under such culture conditions, HB9$^+$ cells were present for at least 5 weeks, although the number began to decrease after 3 weeks. Cells that expressed choline acetyltransferase (ChAT) appeared 3 weeks after the neuroectodermal cells were plated for motoneuron differentiation, and the number of ChAT$^+$ cells increased steadily for up to 7 weeks, the longest culture period analyzed in this study (**Fig. 4a,b**). All ChAT$^+$ cells were βIII-tubulin$^+$ neurons (**Supplementary Fig. 3** online). The ChAT$^+$ cells were largely localized to the cluster (**Fig. 4a**), corresponding to the localization of the HB9$^+$ cells. These cells were mainly multipolar cells with large somas. Most of the ChAT$^+$ cells were 15–20 μm in diameter, with some as large as 30 μm (**Fig. 4a–c**). We observed coexpression of HB9 in the nuclei and ChAT in the soma and processes after 3 weeks of culture (**Fig. 4c**). By 7 weeks in culture, most of the ChAT$^+$ cells became negative for HB9 as assessed by immunohistochemistry. Most of the neurons were also positively stained for vesicular acetylcholine transporter (VAChT) (**Fig. 4d**), a functional molecule for packaging acetylcholine in the presynaptic vesicles. Many ChAT$^+$ cells, especially after 5 weeks in culture, were positively labeled for synapsin on cell bodies and processes (**Fig. 4e**). GFAP$^+$ glia were rare within 3 weeks after the neural progenitors were differentiated. They gradually increased in number and the glial fibers lined up with neurites over time, as shown previously[14].

We assessed functional maturation using electrophysiological techniques (n = 28 cells). The mean resting potential was −36.9 ± 2.6 mV, and input resistance was 920 ± 57 MΩ. Single action potentials (**Fig. 4f,i**) or decrementing trains (**Fig. 4fii**) were elicited by depolarizing current steps (0.15–0.2 nA × 1 s) in 11 of 13 neurons tested. Spontaneous action potentials triggered by spontaneous depolarizing synaptic inputs were also observed (**Fig. 4g**). Although not all cells survived recording and subsequent immunohistochemical analysis, double immunostaining with biocytin and ChAT demonstrated that many of the cells from which we recorded were motoneurons (**Fig. 4j**).

Voltage clamp analysis revealed time- and voltage-dependent inward and outward currents consistent with sodium and delayed rectifier potassium currents. Inward currents and action potentials were blocked by 1 μM tetrodotoxin (TTX, n = 3), confirming the presence of voltage-activated sodium channels. Outward currents were not further characterized. We also observed spontaneous synaptic currents (**Fig. 4h**, n = 21 of 23 cells tested). These were reduced in frequency but not eliminated by 1 μM TTX, demonstrating the existence of functionally intact synaptic neurotransmission. With a cesium gluconate–based pipette solution, outward (inhibitory) currents decayed slowly (13.6 ms, n = 10 events) and were blocked by a combination of strychnine and bicuculline, whereas the remaining inward (excitatory) currents decayed rapidly (2.1 ms, n = 17 events) and were blocked by a combination of D-AP5 and CNQX (**Fig. 4h,i**), demonstrating that inhibitory (GABA/glycine) and excitatory (glutamate) neurotransmission was occurring, as in the intact spinal cord[23]. Thus, the *in vitro*–generated motoneurons were electrophysiologically active and formed functional synapses with neighboring neurons.

We performed further functional analysis using motoneuron-myotube cocultures. Four days after the ventralized neural progenitors were plated onto C2C12 myocytes, synapsin-expressing neurites made contact with forming myotubes. We observed acetylcholine receptor clustering[24], indicated by patched α-bungarotoxin (BTX) staining, on the myotubes immediately adjacent to the axons (Fig. 4k,l). By 2 weeks of coculture, neurites were very extensive and the receptor clustering was more obvious (Fig. 4m,n). Confocal analysis confirmed the apposition of synapsin$^+$ axons and the α-BTX–labeled acetylcholine receptors (Fig. 4n). Dual staining indicated that the myotubes with clustered receptors were innervated by ChAT$^+$ fibers (Fig. 4o). Myocytes or myotubes that did not contact or come close to axons in cocultures (Fig. 4k) or in myocyte culture alone (data not shown) did not show obvious receptor clustering. After 3 weeks of coculture, contraction of the myotubes was observed in the coculture group but not in the cultures of C2C12 myocytes alone. These results indicate that the hES cell–generated motoneurons induce acetylcholine receptor clustering and establish functional neuromuscular transmission.

The present study demonstrates that functional motoneurons can be efficiently generated from hES cells through induction of neuroectoderm, specification and/or caudalization by retinoic acid during the late phase of neuralization, and subsequent differentiation to postmitotic motoneurons in the presence of the ventralizing morphogen SHH. By dissecting the process of neuroectodermal differentiation, we have discovered that specification of early-born projection neurons such as spinal motoneurons requires treatment with morphogens like retinoic acid before precursors become Sox1-expressing neuroectodermal cells.

Although the roles of Pax6 and Sox1 in human neuroectodermal specification are still not completely understood, the functional significance of the two stages of neuralization is becoming clearer. Only the Pax6$^+$ early neuroectodermal cells can be efficiently respecified to a more caudal fate in response to retinoic acid and subsequently differentiated to motoneurons. Considering that Sox1 is expressed by neural plate epithelia during neural tube closure[15], the fact that the forebrain Sox1$^+$ neuroepithelial cells cannot be respecified agrees with the process of *in vivo* neuronal differentiation from progenitors in the neural tube, which are regionally specified[21].

From the standpoint of stem cell biology, ES cells need to be neuralized and then regionalized to progenitors before differentiation into specialized neurons, as demonstrated by the stepwise differentiation of midbrain dopaminergic neurons from mouse ES cells[10,11]. However, these processes are not simply linear but perhaps overlapping. Both the Pax6- and Sox1-expressing neuroectodermal cells can differentiate into large neurons and express transcription factors, such as Islet1 and Lim3, that are associated with motoneuron development[1,19]. But only neuroectodermal cells treated with retinoic acid at an early not a late stage express a balanced level of class I and class II homeodomain genes, leading to the specification of spinal motoneurons. Consequently, the *in vitro*–produced motoneurons possess the key motoneuron transcription factor HB9 and the correct neurotransmitter, transmitter transporter, electrophysiological properties and neuromuscular transmission. Thus, it is necessary to couple the intrinsic program of precursor cells with appropriate morphogens to generate neurons with correct positional identity, and other phenotypes. From the standpoint of both stem cell biology and developmental biology, it is not difficult to understand why brain-derived neuroepithelial cells do not normally generate projection neurons of a different regional identity, particularly after expansion[25].

FGF2 is the most commonly used mitogen for expanding neural precursors. We exposed the hES cell–derived precursors to FGF2 for biotechnological purposes, to increase the population of neuroepithelia, even though exogenous FGF2 is not necessary for neuroepithelial induction. However, continued exposure to FGF2 suppressed the expression of SHH and skewed the expression of class I and class II genes, and subsequently inhibited motoneuron differentiation. Similarly, prolonged exposure of mouse neural progenitor cells to FGF2 disturbed regulation of normal dorsoventral identity[26]. This may explain why FGF2-expanded brain stem cells do not generate large projection neurons, apart from changes in their intrinsic program.

The present study, together with a previous study on motoneuron differentiation from mouse ES cells[12], suggest that fundamental biological principles learned from intact animal studies may be recapitulated *in vitro*, reinforcing the notion that hES cells offer a way to dissect mechanisms of early development in humans, an otherwise inaccessible system[27]. Our study, however, also reveals some unique aspects of neural differentiation from hES cells. The uniform expression of Pax6 in human neuroepithelial cells before Sox1 contrasts with the patterned expression of Pax6 after Sox1 expression in other animals, including mouse. Species differences have been reported in the original derivation of hES cells in terms of growth requirements and expression of cell surface markers[13] and are corroborated by a recent comparison of gene expression profiles in mouse and human ES cells[28]. Hence, one should not assume that information gained from animal studies and protocols established for murine ES cell differentiation can be directly translated to hES cells. hES cells also appear to be more sensitive to morphogens in motoneuron differentiation than are mouse ES cells, as much lower concentrations of morphogens are required for hES cells (0.001–0.1 μM retinoic acid and 50–500 ng/ml SHH) than for mouse ES cells (0.1–2 μM retinoic acid and 6,000 ng/ml SHH)[12]. This, however, could be due to differences in the culture conditions[29]. Technical differences may also account for other differences observed in motoneuron phenotypes. Motoneurons generated in this study express homeodomain protein HoxC8, which is expressed by cells in thoracic regions, whereas motoneurons generated from mouse ES cells[12] have a relatively restricted cervical phenotype. One possible explanation is that hES cells are neuralized by treatment with FGF2, a cytokine that itself possesses caudalizing activity[30], and then treated with retinoic acid. In the earlier study, mouse ES cells were neuralized and caudalized by retinoic acid alone[12]. It is possible that FGF and retinoic acid collaborate in promoting posteriorization.

Functional motoneurons generated from a renewable source of hES cells may be useful for screening pharmaceuticals targeting motoneuron-related disorders, such as amyotrophic lateral sclerosis. These cells may also one day provide replacement motoneurons for applications in patients with motoneuron diseases or spinal cord injury.

METHODS

Culture of hES cells. hES cells (lines H1 and H9, passages 19 to 42) were cultured and passaged weekly on a feeder layer of irradiated embryonic mouse fibroblasts as described[13]. Differentiated colonies were physically removed before passaging and the undifferentiated state of ES cells was confirmed by typical morphology and expression of Oct4 and SSEA4.

Culture of neuroectodermal cells. The procedure for generating neuroectodermal cells from hES cells was described previously[14]. Briefly, ES cell colonies were detached from the feeder layer and grown in the ES cell growth medium in suspension as cell aggregates for 4–6 d. The ES cell aggregates were then adhered to a substrate in a neural induction medium consisting of F12/DMEM, N2 supplement and heparin (2 μg/ml) with or without FGF2 (20 ng/ml). By about 10 d after ES cell differentiation, cells in the center of each colony differentiated into neuroectodermal cells, displaying small columnar

morphology followed by organization of the columnar cells into neural tube–like rosettes after an additional 4–5 d. These neuroectodermal cells were isolated from surrounding nonneural cells through differential response to dispase treatment and a 3–5 d differential adhesion/expansion in the same neural induction medium[14].

Culture of motoneurons. For motoneuron induction, hES cell–derived neuroectodermal cells were first treated with retinoic acid (0.001–1 μM) and/or FGF2 (1–100 ng/ml) at different developmental stages for caudalization (see results). The posteriorized neuroectodermal cells were then differentiated into motoneurons on ornithine/laminin-coated coverslips in a neuronal differentiation medium, which consisted of neurobasal medium (Gibco), N2 supplement, and cAMP (Sigma, 1 μM) in the presence of retinoic acid (0.1 μM) and SHH (50–500 ng/ml, R&D) for one week. After that, BDNF, GDNF and IGF1 (10 ng/ml, PeproTech Inc.) were added to the medium and the concentration of SHH was reduced to 50 ng/ml.

Coculture of motoneurons and myocytes. C2C12 myoblasts were purchased from American Type Culture Collection (ATCC), cultured with DMEM (ATCC) containing 4.5 g/l glucose, 4 mM L-glutamine, 1.0 mM sodium pyruvate and supplemented with 10% fetal bovine serum. After 3–5 d differentiation, multinucleated myotubes began to form.

For coculture of motoneurons and myocytes, the posteriorized neuroectodermal cells were differentiated for 1–2 weeks in the presence of retinoic acid and SHH. Either these ventral progenitor cells were plated on top of myotubes or the dissociated C2C12 myoblasts were plated onto the neuronal progenitor cells in the neural differentiation medium consisting of neurobasal medium, N2 supplement, cAMP, BDNF, GDNF and IGF1.

Immunocytochemistry and microscopy. Immunohistochemical staining was performed as previously described[14]. Primary antibodies used in this study included polyclonal antibodies against βIII-tubulin (Covance Research Products, 1:2000), nestin (Chemicon, 1:750), Sox1 (Chemicon, 1:1000), synapsin I (Calbiochem, 1:500), ChAT (Chemicon, 1:100), VAChT (Chemicon, 1:1000), Islet1/2 (S. Pfaff), Otx2 (F. Vaccarino) and Olig2 (M. Nakafuku). Antibodies against MNR2 or HB9 (81.5C10), Islet1 (40.2D6), Lim3 (67.4E12), Pax6, Pax7 and Nkx2.2, were purchased from Developmental Studies Hybridoma Bank (DSHB), and anti-HoxC8 from Covance Research Products (1:200). For identification of electrophysiologically recorded cells, cells filled with biocytin (Molecular Probes) were labeled with streptavidin-FITC (Sigma, 1:200) and stained for ChAT. For labeling acetylcholine receptors, coverslip cultures were incubated with Alexa Fluor 594 conjugated α-BTX (Molecular Probes, 1:500) at 20 °C for 30 min. Images were collected using a Spot digital camera mounted onto a Nikon fluorescent microscope 600 (Fryer Inc.) or a confocal microscope (Nikon). Orthogonal confocal images were rendered via Nikon-C1 image software. The specificity of antibodies against motoneuron transcription factors and homeodomain proteins, which were originally developed against non-primate tissues, were verified in embryonic (E34 or E36) rhesus monkey spinal cord and brain tissues (provided by the Wisconsin Primate Research Center). In each set of experiments, positive controls were set up using embryonic mouse (E13) and monkey (E34 or E36) spinal cord and brain tissues and negative controls lacking primary or secondary antibodies were included.

Quantification. The population of HB9-expressing cells among total differentiated cells (Hoechst labeled) were counted in two ways. One was to capture images using a digital camera and individual, positively stained cell nuclei were counted by a person who was blind to experimental groups using the Metamorph software (Universal Imaging Corporation). The second method was stereological measurement using an automated stage movement operated by Stereo Investigator software (MicroBrightField Inc.). In this method, an area to be measured was outlined by a tracer, with the number of counting frames preset so that the scope sampled the measuring sites randomly. For counting areas with overlapping cells, the microscope was preset to move up and down to focus on the positive cells in different layers and the total cell number in the cluster was estimated by the software. We quantified the HB9 positive cells in the cluster and the outgrowth area separately. Both methods produced very similar results. Three to four coverslips in each group were counted and data were expressed as mean ± s.d.

RT-PCR assays. Total RNA was extracted from hES cell–derived neuroectodermal cells at different stages and motoneuron differentiation cultures using RNA STAT-60 (TEL-TEST, INC.). cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen) according to the supplier's protocol and was used as templates for the PCR reaction. PCR reactions were performed in 15-μl mixture containing cDNA, primers and 1× PCR Master mix (Promega). The following primers were used: HoxC8, 5′-TTTATGGGGCTCAG CAAGAGG-3′, 5′-TCCACTTCATCCTTCGGTTCTG-3′, 318 bp; HoxC5, 5′-TC GGGGTGCTTCCTTGTAGC-3′, 5′-TTCGTGGCAGGGACTATGGG-3′, 290 bp; HoxB6, 5′-AACTCCACCTTCCCCGTCAC-3′, 5′-CTTCTGTCTCGCCG AACACG-3′, 340 bp; Otx2, 5′-CAACAGCAGAATGGAGGTCA-3′, 5′-CTGGGT GGAAAGAGAAGCTG-3′, 429 bp; HoxB1, 5′-TCAGAAGGAGACGGAGGC TA-3′, 5′-GTGGGGGTGTTAGGTTCTGA-3′, 218 bp; GAPDH, 5′-ACCACAGT CCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′, 450 bp; Olig2, 5′-AAGGAGGCAGTGGCTTCAAGTC-3′, 5′-CGCTCACCAGTCGCTTCATC-3′, 315 bp; Nkx2.2, 5′-TGCCTCTCCTTCTGAACCTTGG-3′, 5′-GCGAAATCTGC CACCAGTTG-3′, 337 bp. Irx3, 5′-AAGAACGCCACCAGGGAGAG-3′, 5′-TTG GAGTCCGAAATGGGTCC-3′, 473 bp; Pax6, 5′-GGCAACCTACGCAAGATG GC-3′, 5′-TGAGGGCTGTGTCTGTTCGG-3′, 459 bp; SHH, 5′-CCAATTACAA CCCCGACATC-3′, 5′-CCGAGTTCTCTGCTTTCACC-3′, 339 bp; Nkx6.1, 5′-ACACGAGACCCACTTTTTCCG-3′, 5′-TGCTGGACTTGTGCTTCTTCAA C-3′, 335 bp; Sox1, 5′-CAATGCGGGGAGGAGAAGTC-3′, 5′-CTCTGGACCA AACTGTGGCG-3′, 464 bp.

Electrophysiology recording. Electrophysiological properties of hES cell–derived motoneurons were investigated in cultures differentiated for 5–6 weeks using whole-cell, patch-clamp recording techniques[23]. Tetrodotoxin (TTX, 1 μM; Sigma), bicuculline (20 μM; Sigma), strychnine (5 μM; Sigma), D-2-amino-5-phosphonovaleric acid (AP-5, 40 μM; Sigma) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; RBI) were applied in the bath solution to confirm the identity of voltage-activated or synaptic currents. For some experiments, 1% biocytin was added to the recording solution to fill the cells after recording. Current-clamp and voltage-clamp recordings were made using a MultiClamp 700A amplifier (Axon Instruments). Signals were filtered at 4 kHz, sampled at 10 kHz using a Digidata 1322A analog-digital converter (Axon Instruments), and acquired and stored on a computer hard disk using commercially available software (pClamp9, Axon Instruments). Access resistance was typically 8–15 MΩ and was compensated by 50–80% using amplifier circuitry. The resting membrane potential and spontaneous and depolarization-evoked action potential properties were examined in current-clamp mode. Spontaneous excitatory (inward) and inhibitory (outward) synaptic currents were characterized in voltage-clamp mode. For analysis, events were detected using a template detection algorithm (Mini Analysis Program 5.6.28; Synaptosoft). Synaptic current decays were fitted to a monoexponential function using the Levenberg-Marquardt algorithm. Data are presented as mean ± s.e.m.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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